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Adrenergic Receptors Mediating Pigment Movements in Chromatophores of the Medaka, *Oryzias latipes*

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Contents

I. General introduction

Many teleost fishes rapidly change their body tints or color patterns in response to background hues or various stresses. Such changes of tints and color patterns are called physiological color changes and occur due to translocation of pigment granules within specialized colored cells, chromatophores, in scales and fins of the animals (cf. Parker, 1948; Bagnara and Hadley, 1973; Fujii and Oshima, 1986).

Three types of chromatophores, i.e. melanophores xanthophores and leucophores, are usually found to be present in scales of the medaka, Oryzias latipes. Among these, the cells mainly responsible for the body tints of animals are melanophores and leucophores. Both centrifugal translocation of pigment granules within melanophores (dispersion of melanosomes) and centripetal migration of pigment within leucophores (aggregation of leucosomes) bring about a darkening of body tint, while the reverse, i.e. melanosome aggregation of melanophores and leucosome dispersion of leucophores, cause body paling.

Previous physiological and histological investigations have suggested that both melanophores and leucophores of the medaka receive the same adrenergic innervation and that the neurotransmitter released is norepinephrine (Iwata et al., 1981; Iga, 1983; Yamada et al., 1984). In physiological saline, melanophores usually maintain a fully melanosome-dispersed state and

leucophores are in a fully leucosome-aggregated state. Nerve-mediated stimuli, such as electric currents and potassium ions, or exogenous norepinephrine induce melanosome-aggregation of melanophores, while at the same time, the stimuli cause leucosome dispersion of leucophores (Miyoshi, 1957; Iga, 1969, 1978).

On the other hand, pharmacological researches on medaka chromatophores have revealed that adrenergic receptors which mediate aggregation of melanosomes in melanophores are alpha in nature and that those which mediate dispersion of leucosomes in leucophores are beta in nature (Iga, 1968; Obika, 1976; Iga et al., 1977; Yamada, 1980). However, subsequent investigations have demonstrated that alpha adrenergic receptors are also present in leucophore, which mediate aggregation of leucosomes (Iga, 1979) and that melanophores also possess beta adrenergic receptors, whose stimulation causes melanosome dispersion (Komatsu and Yamada, 1982).

Thus, notwithstanding the findings that both melanophores and leucophores of the medaka receive the same adrenergic innervation and that these chromatophores possess both alpha and beta adrenergic receptors, they respond to nerve stimuli or exogenous norepinephrine in completely opposite directions to each other. This implies that the affinities of adrenergic receptors for the neurotransmitter,

norepinephrine, differ between melanophores and leucophores.

The aim of the present study was to analyse how the receptor mechanism is involved in the regulation of such different responses of medaka chromatophores to identical stimuli.

In mammalian adrenergic tissues, depending on the affinities for specific adrenergic agonists and antagonists, both alpha and beta adrenergic receptors are classified into two subtypes, i.e. alpha-1 and alpha-2 (Starke, 1981; Johannson, 1984) and beta-1 and beta-2 (Lands et al., 1967; Petrack and Andrew, 1976; Barnett et al., 1978; Minneman et al., 1979), respectively.

Therefore, in the present experiment, according to the criteria for mammalian tissues, subtypes of adrenergic receptors in both melanophores and leucophores of the medaka were investigated. The results demonstrated that the subtypes of alpha adrenergic receptors which mediate dispersion of pigment granules within melanophores and leucophores are beta-2 and beta-1, respectively (Chapter III), and the subtypes of alpha receptors mediating aggregation of pigment granules within both of these chromatophores are alpha-2 in nature (Chapters IV and V) and showed that the observed difference between melanophores and leucophores in response to identical stimuli is due to such differences in the subtypes of receptors in the cells.

II. Materials and methods

Preparation and procedure

Experiments were performed with chromatophores in scales of the medaka, Oryzias latipes (wild type). The scales were isolated from antero-dorsal region of the body trunks of the animals. To expose chromatophores directly to drugs used, isolated scales were preliminarily incubated in 10 mM EDTA (ethylenediaminetetraacteic acid) in physiological saline (128 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, pH 7.3 with 5.0 mM Tris-HCl) for 60 min at 4.0°C and agitated to remove the epithelial cells which overlie the chromatophores. The chromatophores used here were exclusively the denervated ones to exclude any indirect influence of the drugs through pre-junctional elements. Denervation was secured after maintaining the isolated scales in physiological saline for more than 15 hr at room temperature (Yamada, 1980).

The response of melanophores was measured photoelectrically by a CdS photoconductive cell mounted on the eye piece of microscope according the method described by Nagahama and Katayama (1982) and recorded on an eletronic polyrecorder (TOA, EPR-221A). The magnitude of the melanosome-aggregation response was expressed as a percentage of the full change in light transmittance, using a value for maximal aggregation as 100 and that for full dispersion as zero.

For observation of leucophores, reflected light through a dark-field epi-illumination microscope

(Olympus, BHA-NE) was used. The response of the leucophores was represented by percentage changes in apparent length of a given process of the cells, which was measured with the aid of an ocular micrometer. The length of the process at full leucosome dispersion was taken as 100 and that at the fully aggregated state as zero.

Drugs used

Adrenergic agonists and antagonists used were as follows:

Catecholamines. l-Norepinephrine hydrochloride (Sigma), l-epinephrine bitartrate (Tokyo Chemical) and dl-isoproterenol hydrochloride (Tokyo Chemical).

Beta agonists. Salbutamol (Sigma) and terbutaline sulphate (Fujisawa Pharmaceutical).

Beta antagonists. Propranolol hydrochloride (Sigma), metoprolol tartrate (Ciba-Geigy) and atenolol (Sigma).

Alpha agonists. Methoxamine hydrochloride (Nippon Shinyaku), l-phenylephrine hydrochloride (Sigma), tetrahydrozoline hydrochloride (Sigma), clonidine hydrochloride (Sigma), naphazoline hydrochloride (Sigma), oxymetazoline hydrochloride (Sigma) and tramazoline hydrochloride (Boehringer Ingelheim-Tanabe).

Alpha antagonists. Phentolamine mesylate (Ciba-Geigy), corynanthine hydrochloride (Sigma), prazosin hydrochloride (Tokyo Chemical) and yohimbine hydrochloride

(Sigma).

All the drugs were prepared for stocks at 1 mM in deionized water except norepinephrine hydrochloride and epinephrine bitartrate, which were dissolved in 0.5% sodium metabisulphite, and prazosin hydrochloride which was dissolved in 1% ethanol. Alpha-MSH (melanophore-stimulating hormone) was also used, which was dissolved and stored in 100 mM aqueous solution of ascorbic acid at 50 μM. Each stock solution was diluted with physiological saline immediately before use.

All the experiments were carried out at 20-25°C.

Statistics

Statistical analyses were performed with Student's t test and $p < 0.01$ was used as the level of significance.

III. Subtypes of beta adrenergic receptors mediating pigment dispersion in chromatophores

Dermal melanophores of teleost fishes receive adrenergic innervation (Falck et al., 1969; Reed and Finnin, 1972; Yamada et al., 1984; also cf. Parker, 1948; Bagnara and Hadley, 1973; Fujii and Oshima, 1986) and both electric currents and potassium ions induce melanosome aggregation within the cells through stimulation of their nervous elements (Fujii, 1959; Fujii and Novales, 1969; Kinosita and Ueda, 1970; Fujii and Miyashita, 1975; Iga, 1975). Moreover, it has been suggested that the released neurotransmitter is norepinephrine (Fujii, 1961; Scheline, 1963; Fujii and Novales, 1972) and that adrenergic receptors mediating the aggregation of melanosomes are of alpha nature (Iga, 1968; Grove, 1969; Fernando and Grove, 1974a, b; Fujii and Miyashita, 1975). Meanwhile, recent pharmacological studies have suggested the presence of beta adrenergic receptors which mediate dispersion of melanosomes in the melanophores of the angelfish, Pterophyllum eimekei (Reed and Finnin, 1972) and the guppy, Lebistes reticulutus (Miyashita and Fujii, 1975). Lately, using specific radioligands, Komatsu and Yamada (1982) have demonstrated the localization of beta receptors autoradiographically in melanophores of the medaka.

In contrast, leucophores, another fish chromatophores, have been known to behave in quite the opposite way to melanophores; they respond to both nerve-mediat-

ed stimuli, i.e. electric currents and potassium ions, and exogenous norepinephrine with leucosome dispersion through the mediation of beta adrenergic receptors (Miyoshi, 1952; Iga et al., 1977; Yamada, 1980). In spite of the difference in the response to identical stimuli, it has been suggested that melanophores and lecuophores receive the same sympathetic postganglionic fibers (Iwata et al., 1981; Iga, 1983; Yamada et al., 1984). Hence, we may wonder whether identical stimuli can induce such opposite responses by these chromatophores.

In mammalian adrenergic tissues, beta receptors in cells are classified into two subtypes, i.e. beta-1 and beta-2, accroding to their affinity for specific agonists and antagonists (Lands et al., 1967; Petrack and Andrew, 1967; Barnett et al., 1978; Minneman et al., 1979). In fish species, based on the potency of several sympathomimetic amines to cause dispersion of leucophore pigment, Yamada (1980) has suggested that adrenergic receptors which mediate the cell response of the medaka are beta-1 in nature. However, no such attempts have been made on the subdivision of beta receptors in fish melanophores. Therefore, in the present experiment, to define more precisely the nature of subtypes of beta adrenergic receptors in melanophores and leucophores, the responses of these chromatophores to specific agonists and antagonists of beta receptors

were investigated. The results have demonstrated that the adrenergic receptors mediating melanosome-dispersion response of melanophores are beta-2 and those mediating leucosome-dispersion response of leucophores are beta-1.

Results

Effects of catecholamines and propranolol on melanophores

First, to determine the melanosome-dispersing actions of catecholamines on melanophores, the effects of norepinephrine, epinephrine and isoproterenol were examined under the influence of propranolol, a beta adrenergic antagonist (Fig. 1). Norepinephrine, with the concentration at 1 nM, alone induced a moderated melanosome aggregation, i.e. about 40% of the full aggregation which was obtained with the amine at 10 μM (Fig. 1a). Propranolol at 1 μM had almost no effect on the response of melanophores to norepinephrine. However, the drug considerably augmented the response of cells to both 5 nM epinephrine and 10 μM isoproterenol, while each of the amines induced about a half maximal aggregation of melanosomes (Fig. 1b and c).

Figure 2 shows the relationship of the magnitude of melanosome-aggregation response of melanophores to the concentration of catecholamines in the presence or absence of propranolol. Under the influence of propranolol, the degree of aggregation response to norepinephrine slightly decreased and the concentration-response curve shifted to higher concentration of the amine (Fig. 2a), but the difference was insignificant ($P > 0.60$). On the other hand, the curves for both

epinephrine and isoproterenol shifted to lower concentrations in the presence of propranolol and effective concentrations of the amines to cause 50% aggregation (EC_{50}) of melanosomes decreased significantly ($P<0.005$) from 2.1 to 1.4 nM and from 8.6 to 3.1 μM, respectively (Fig. 2b and c).

Effects of beta-2 agonists

The effects of beta-2 agonists, salbutamol which is also known to be a beta-1 antagonist and terbutaline were examined on responses of both melanophores and leucophores to catecholamines (Fig. 3). Norepinephrine at 10 nM induced a nearly full aggregation of melanosomes, but the level of aggregation decreased to about 60% under the influence of 1 μM salbutamol (Fig.3a). The effect of the drug was completely abolished by the presence of 1 μM propranolol. Salbutamol alone had no effect on melanophores as can be seen in the figure. On the other hand, leucophores responded 100 nM isoproterenol with full dispersion of leucosomes as well as to 10 μM norepinephrine and the response was markedly suppressed by the presence of salbutamol, though the drug had no intrinsic effect on the cells (Fig. 3b).

Terbtaline, another beta-2 agonist used, at 10 μM had a similar effect on both melanophores and leucophores to that of salbutamol (Fig. 4). Thus, terbutaline reduced both the melanosome-aggregation response

of melanophores to norepinephrine (Fig. 4a) and leucosome-dispersion response of leucophores to isoproterenol (Fig. 4b), respectively.

Such effects of beta-2 agonists were reversible and the chromatophores could respond to catecholamines as usual after rinsing with normal saline.

The relation between the magnitude of the chromatophore response and the concentration of catecholamines is shown in Figure 5. As is apparent from the figure, concentration-response curves for both melanophores and leucophores shifted to higher concentrations of the amines in the presence of beta-2 agonists. The EC_{50} of norepinephrine for aggregation of melanosomes increased from 2.7 to 9.4 nM ($\underline{P} < 0.01$) and to 13.7 nM ($\underline{P} < 0.001$) in the presence of 1 μM salbutamol and 10 μM terbutaline (Fig. 5a), while the EC_{50} of isoproterenol for leucosome dispersion increased from 24.8 to 93.3 nM ($\underline{P} < 0.001$) and to 158.0 nM ($\underline{P} < 0.01$) under the influence of the drugs (Fig. 5b), respectively. The effects of beta-2 agonists were concentration-dependent and the responses of both melanophores to 10 nM norepinephrine and leucophores to 100 nM isoproterenol were totally depressed by the presence of the drugs at concentrations higher than 100 μM.

Effects of beta-1 antagonists

The effects of specific beta-1 antagonists, meto-

prolol and atenolol, were examined. These drugs had virtually no effect on the pigmentary state of melanophores which had been dispersed in normal saline or aggregated by norepinephrine. Then, the effects were examined on norepinephrine-treated aggregated melanophores in the presence of isoproterenol (Figs. 6a and 7a). Isoproterenol at 100 nM induced a marked decrease in the level of the melanophore response to 10 nM norepinephrine, but the action was never affected by the presence of 0.5 μM metoprolol or 1 μM atenolol. On the other hand, although the antagonists had no intrinsic effect on leucophores, they significantly ($\underline{P} < 0.01$) inhibitied the leucosome-dispersion response of the cells to isoproterenol (Figs. 6b and 7b). The inhibitory effects of both antagonists on leucophores could be removed completely through washing in normal saline.

Figure 8 shows the influence of beta-1 antagonists on the concentration-response curve for leucosome-dispersion response of the leucophores to isoproterenol. The EC_{50} for the control curve was 14.0 nM, but it shifted to 192.0 nM ($\underline{P} < 0.001$) and to 63.4 nM ($\underline{P} < 0.01$) in the presence of 0.5 μM metoprolol and 1 μM atenolol, respectively. The effects of these drugs were also concentration-dependent and the leucophore response to 100 nM isoproterenol was abolished completely either by metoprolol at concentrations higher than 10 μM or by atenolol higher than 100 μM.

Discussion

It has been known that catecholamines induce the aggregation of melanosomes within fish melanophores through the mediation of alpha adrenergic receptors (Iga, 1968; Grove, 1969; Fernando and Grove, 1974a, b; Fujii and Miyashita, 1975). The present results indicated that all the catecholamines examined also have a melanosome-aggregating action of melanophores of the medaka and that propranolol, a beta adrenergic antagonist, augument the action of the amines except that of norepinephrine. This suggests that usually both epinephrine and isoproterenol have a melanosome-dispersing action on melanophores in addition to the melanosome-aggregating action and that propranolol augments the aggregation response of melanophores to catecholamines through inhibition of beta adrenergic receptors which mediate melanosome dispersion within the cells. Moreover, the present results demonstrated that the beta-2 agonists, salbutamol and terbutaline, competitively suppressed the melanophore response to norepinephrine. This indicates that the beta adrenergic receptors which mediate melanosome dispersion within melanophores are beta-2. Thus, salbutamol and terbutaline antagonistically inhibit the melanosome-aggregating action of norepinephrine by their melanosome-dispersing actions on the cells through mediation of beta-2 receptors. Al-

though the effects of beta-2 antagonists were not examined here, the finding that beta-1 antagonists did not interfere with the dispersion response of melanophores to isoproterenol substantiates indirectly the presence of beta-2 receptors in the cells.

With regard to the leucophores of the medaka, adrenergic receptors which mediate leucosome dispersion are suggested to be beta-1 (Yamada, 1980). The present results showed this, i.e. that both salbutamol and terbutaline, beta-2 agonists (and which also are beta-1 antagonists), themselves had no leucosome-dispersing effect on the cells, but inhibited the dispersion response of the cells to isoproterenol. The beta-1 antagonists, metoprolol and atenolol, also competitively inhibited the leucophore response to isoproterenol, which substantiate the idea that adrenergic receptors mediating leucosome dispersion within leucophores are subtype beta-1.

Rapid paling in body tint of teleost fishes is mainly due to aggregation of melanosomes within melanophores. In some fish species, leucophores are also concerned with the paling response. In vivo studies on Fundulus heteroclitus (Foster, 1933; Fries, 1942) and on Bachygobius soporaor (Fries, 1958) have shown that when animals are placed on a white background, leucophores in the scales and fins respond with dispersion of leucosomes, while melanophores respond with aggre-

gation of melanosomes. A similar opposite response in two chromatophores has been demonstrated in vitro on isolated scales of Fundulus to exogenous epinephrine (Odiorne, 1933) and on those of the medaka to potassium ions (Miyoshi, 1952).

Recent investigations suggest physiologically and histologically that both melanophores and leucophores of the medaka are innervated by the same adrenergic fibers and that a putative neurotransmitter released is norepinephrine (Iwata, et al., 1981; Iga, 1983; Yamada et al., 1984). Then, the question arises how melanophores and leucophores usually respond in different ways to identical stimuli, such as electric currents, potassium ions or exogenous norepinephrine. Norepinephrine is generally accepted to be a beta-1 agonist as well as an alpha agonist (Lands et al., 1967; Carlsson and Hedberg, 1967; Minneman and Molinoff, 1980). As shown in the present study, the beta receptors in the medaka melanophores are not beta-1 in subtype but are beta-2, so the neurotransmitter or exogenous norepinephrine does not act on them but acts preferably on alpha receptors to induce aggregation of melanosomes. On the other hand, beta receptors of leucophores are exclusively beta-1 and the cells respond to norepinephrine properly with dispersion of leucosomes. Here, it can be said that, through such a difference in the receptor subtypes, melanophores and

leucophores behave *in vivo* and *in vitro*, in quite opposite directions to each other.

Lately, Miyashita *et al*. (1984) have demonstrated the presence of adenosine receptors which mediate melanosome dispersion in guppy melanophores. Moreover, Kumazawa *et al*. (1984) and Kumazawa and Fujii (1984) have indicated that adenosine or ATP is released as a cotransmitter from chromatic nerves of the tilapia, *Sarotgerodon niloticus*. If so, how are beta-2 receptors in melanophores concerned with the dispersion of melanosomes? Miyashita and Fujii (1975) have suggested that the endogenous beta stimulating amine responsible for melanosome dispersion of melanophores is epinephrine and that its source is chromaffin cells in adrenal tissues or in the dermis. The same may be applied to the present case. Endogenous epinephrine may induce dispersion of melanosomes through mediation of beta-2 receptors, but there is no real evidence at present to support this.

In mammalian heart and lung tissues, both beta-1 and beta-2 receptors have been known to exist in the same cells (Lands *et al*., 1967; Barnett *et al*., 1978; Minneman *et al*., 1979; Minneman and Molinoff, 1980). The neurotransmitter of sympathetic nerves is norepinephrine and it acts mainly via beta-1 receptors, while circulating epinephrine may activate beta-2 receptors causing an additional potentiation of the tissue re-

sponses (Carlson and Hedberg, 1976; Minneman et al., 1979; Minneman and Molinoff, 1980).

On the contrary, in cardiac tissues of the frogs, Rana esculenta, R. pipiens and R.temporaria (Stene-Larsen and Helle, 1978; Hancock et al., 1979; Sten-Larsen, 1981) and the teleosts Pleuronectes platessa (Falck et al., 1966) and Salmo gairdneri (Ask et al., 1980), it has been suggested that the main sympathetic neurotransmitter is epinephrine which acts on beta-2 receptors and that beta-1 receptors are less important in the tissues and are activated by circulating norepinephrine.

However, unlike the results demonstrated in the cardiac tissues of the teleosts, the neurotransmitter of the chromatic adrenergic nerves of teleosts is norepinephrine and not epinephrine. At present, wether such a difference in neurotransmitter mechanism between the chromatophore system and cardiac tissues is due to tissue specificity or species specificity is not known.

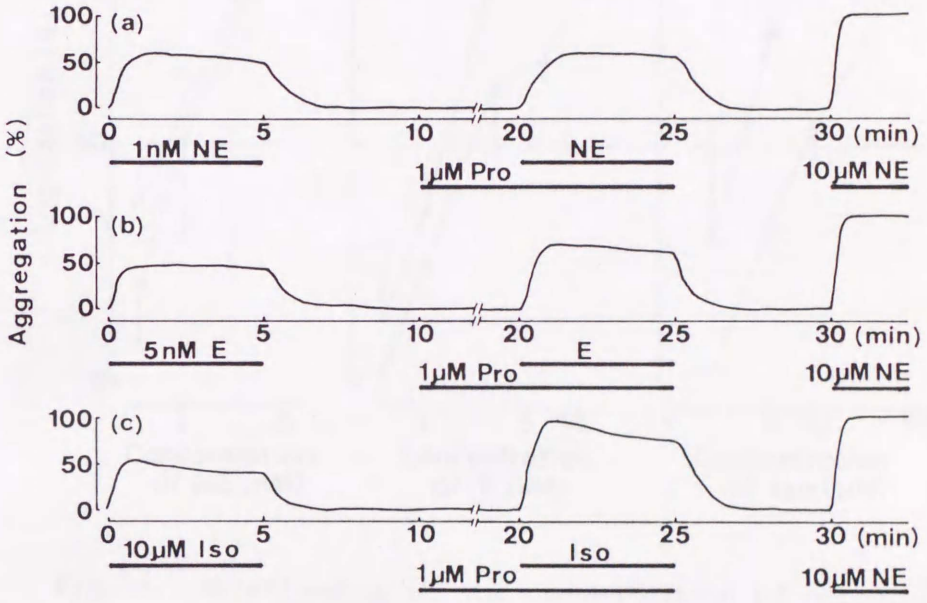

Fig. 1. Typical recordings showing the melanosome-aggregation response of denervated melanophores of the medaka to (a) 1 nM norepinephrine (NE), (b) 5 nM epinephrine (E) and (c) 10 μM isoproterenol (Iso), before and after treatment with i μM propranolol (Pro). Abscissae, time in min. Ordinates, magnitude of response as a percentage of the maximal level of aggregation that was obtained in response to 10 μM NE. Note that both records and time scales are broken at marks for convenience.

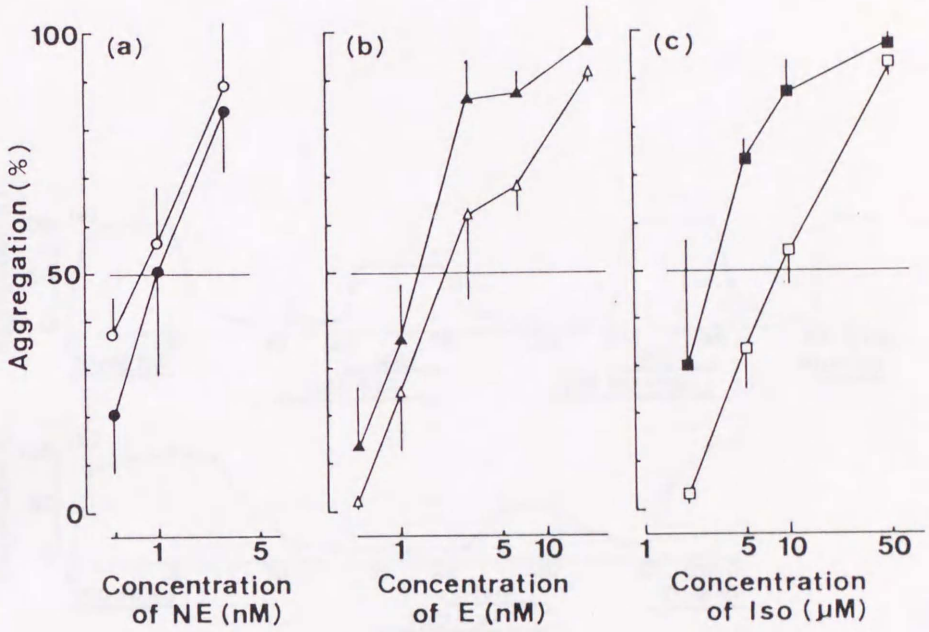

Fig. 2. Relationship of the concentration of norepinephrine (a), epinephrine (b) and isoproterenol (c) to the magnitude of melanosome-aggregation response of the medaka melanophores in the absence (open symbols) and the presence (solid symbols) of 1 μM propranolol. Abscissae, concentration of amines in logarithmic scale. Ordinates, magnitude of aggregation response (%). Each point is the mean of 10 measurements in different animals and vertical bars indicate SDs.

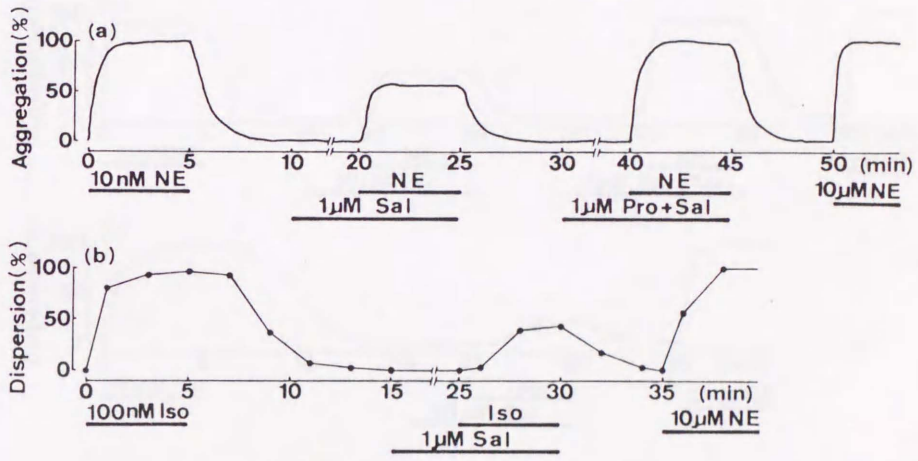

Fig. 3. Typical recordings showing the effects of salbutamol on chromatophores of the medaka. (a) Melanosome-aggregation response of melanophores to 10 nM norepinephrine (NE) under the influence of 1 μM salbutamol (Sal) with or without the presence of 1 μM propranolol (Pro). Abscissa, time in min. Ordinate, magnitude of aggregation response (%). (b) Leucosome-dispersion response of leucophores to 100 nM isoproterenol (Iso) in the presence of 1 μM salbutamol. Ordinate, magnitude of response as a percentage of the maximal level of dispersion which was obtained with 10 μM NE.

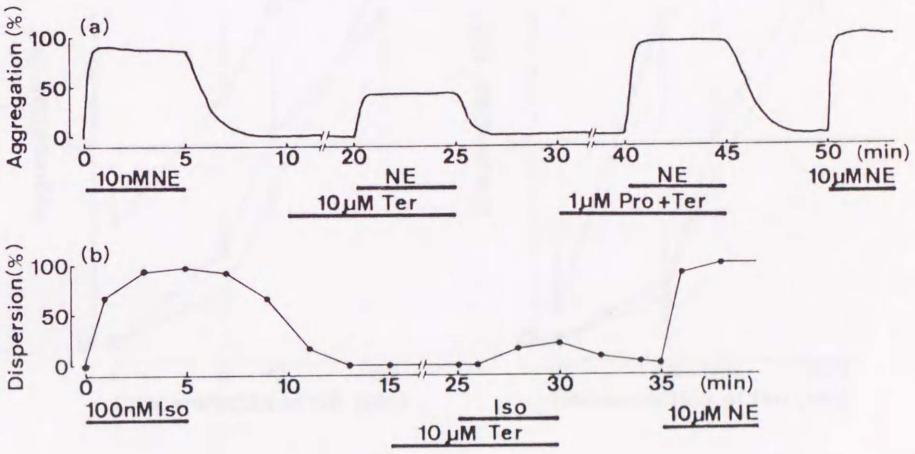

Fig. 4. Typical recordings showing the effects of terbutaline on chromatophores of the medaka. (a) Melanosome-aggregation response of melanophores to 10 nM norepinephrine (NE) under the influence of 10 μM terbuthaline (Ter) with or without the presence of 1 μM propranolol (Pro). (b) Leucosome-dispersion response of leucophores to 100 nM isoproterenol (Iso) in the presence of 10 μM terbutaline. For explanation of abscissae and ordinates for this and Figs. 6 and 7, see legend of Fig. 3.

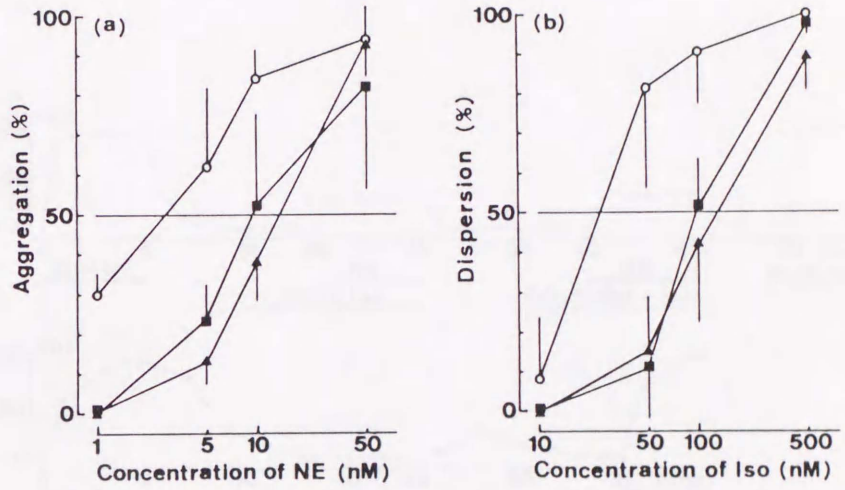

Fig. 5. (a) Relation between the concentration of norepinephrine and the magnitude of aggregation response of melanophores in the absence (open circles) or presence of either 1 μM salbutamol (solid squares) or 10 μM terbutaline (solid triangles). Abscissa, concentration of norepinephrine (NE) in logarithmic scale. Ordinate, magnitude of aggregation response (%). (b) Relationship of the concentration of isoproterenol to the magnitude of dispersion response of leucophores in the absence (open circles) or presence of 1 μM salbutamol (solid squares) or 10 μM terbutaline (solid triangles). Abscissa, concentration of isoproterenol (Iso) in logarithmic scale. Ordinate magnitude of dispersion response (%). Each point in the figure is the mean of 10 measurements in different animals and the vertical bars indicate SDs.

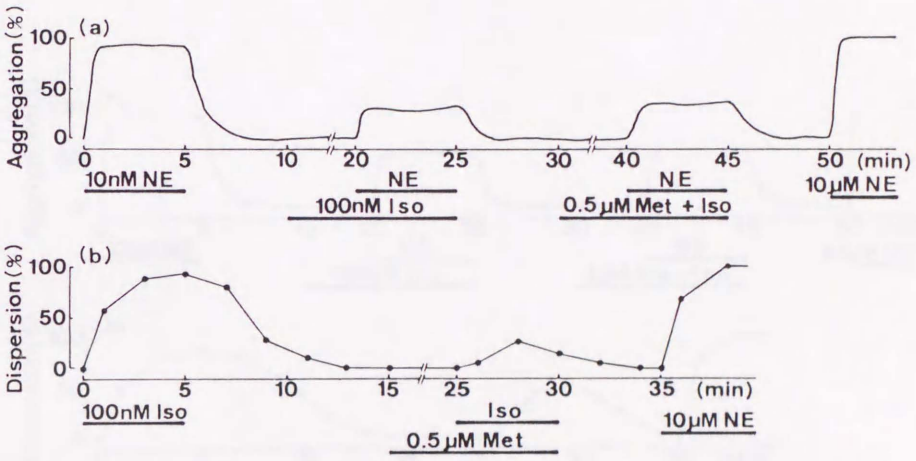

Fig. 6. Typical recordings showing the effects of 0.5 μM metoprolol (Met) on the melanosome-aggregation response of melanophores to 10 nM norepinephrine (NE) under the influence of 100 nM isoproterenol (Iso) (a) and on the leucosome-dispersion response of leucophores to 100 nM isoproterenol (b).

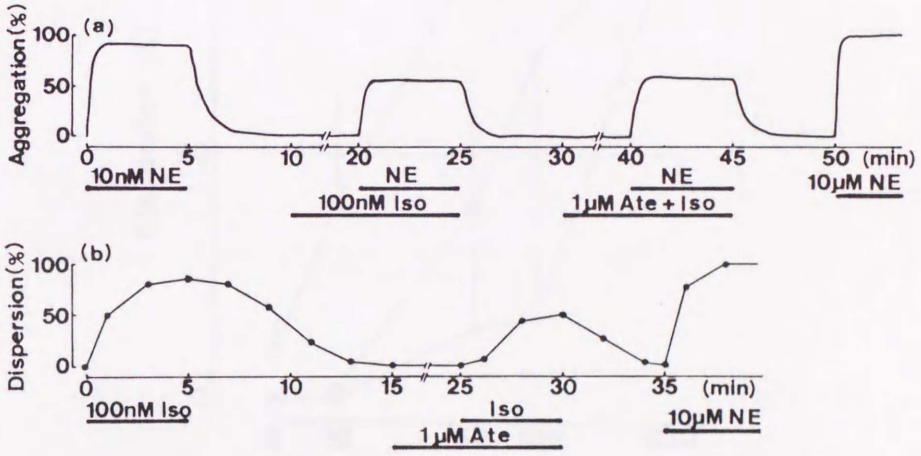

Fig. 7. Typical recordings showing the effects of 1 μM atenolol (Ate) on the melanosome-aggregation response of melanophores to 10 nM norepinephrine (NE) under the influence of 100 nM isoproterenol (Iso) (a) and on the leucosome-dispersion response of leucophores to 100 nM isoproterenol (b).

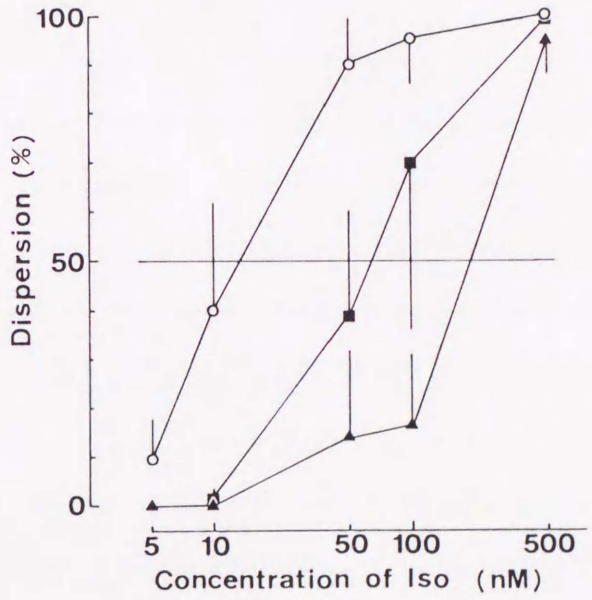

Fig. 8. Relation between the magnitude of leucosome-dispersion response of leucophores and the concentration of isoproterenol without (open circles) or with 0.5 μM metoprorol (solid triangles) or 1 μM atenolol (solid sqares). Abscissa, concentration of isoproterenol in logarithmic scale. Ordinate, magnitude of dispersion response (%). Each point is the mean of 10 measurements in different animals and the vertical bars indicate SDs.

IV. The subtype of alpha adrenergic receptors mediating melanosome aggregation in melanophores

Melanophores in scales and fins of teleost fishes have been shown to receive adrenergic innervation (Jacobowitz and Laties, 1968; Falck et al., 1969; Reed and Finnin, 1972; Fujii and Miyashita, 1975) and the putative neurotransmitter of the chromatic nerve is suggested to be norepinephrine (Fujii, 1961; Scheline, 1963; Fujii and Novales, 1972). Furthermore, nerve stimuli or exogenous norepinephrine have been demonstrated to induce aggregation of melanosomes within melanophores through stimulation of alpha adrenergic receptors (Grove, 1969; Fernando and Grove, 1974a, b; Fujii and Miyashita, 1975). Meanwhile, it has been also shown that dispersion of melanosomes within melanophores occur through mediation of beta adrenergic receptors (Reed and Finnin, 1972; Miyashita and Fujii, 1975; Yamada et al., 1983).

Recent physiological studies on medaka scales have suggested that melanophores of the medaka also receive adrenergic innervation and that the nerve stimulation produces aggregation of melanosomes within the cells through mediation of alpha adrenergic receptors (Iwata et al., 1981; Iga, 1983). Using ^{3}H-norepinephrine, Yamada et al. (1984) have demonstrated autoradiographically the pattern of innervation to melanophores in scales of the medaka and have ascertained that the neurotransmitter released is norepinephrine.

In the preceding chapter, according to the crite-

ria for subclassification of adrenergic receptors in mammalian tissues (Lands et al., 1967; Minneman et al., 1979), it was determined that beta adrenergic receptors which mediate melanosome dispersion of medaka melanophores are beta-2 in the subtype. However, at present, the subtype of alpha adrenergic receptors in medaka melanophores has been not examined. In mammalian tissues, alpha adrenergic receptors which mediate various cell responses are also classified into two subtypes, alpha-1 and alpha-2, depending on the affinities of cells for specific agonists and antagonists (Starke, 1981; Johansson, 1984). In cold-blooded vertebrates, studies on subtypes of alpha adrenergic receptors which participate in the melanosome-aggregation response of melanophores are comparatively few (Berthelsen and Pettinger, 1977; Carter and Shuster, 1982; Andersson et al., 1984). Therefore, in the present experiment, as an aid to analyse the receptor mechanisms involved in the regulation of chromatophore movements, the subtype of alpha adrenergic receptors in the medaka melanophores were investigated using a series of alpha adrenergic agonists and antagonists, in which also the resemblance and difference in the affinity of cell to the adrenergic drugs were examined between the present melanophores and the other heretofore studied cells.

Results

Effects of alpha agonists

All the alpha agonists examined induced a reversible and concentration-dependent melanosome aggregation of melanophores, but the effective concentration and the maximal effect obtained for each agonist were observed to differ considerably.

Figure 9 shows the relationship of the magnitude of melanosome-aggregation response of melanophores to the concentration of agonists. The melanosome-aggregating potency of agonists and the EC_{50}'s estimated from the curves were in the following order: norepinephrine (3.3 nM), epinephrine (8.5 nM), naphazoline (30.0 nM), tramazoline (44.7 nM), clonidine (55.0 nM), tetrahydrozoline (0.15 μM), phenylephrine (37.5 μM), oxymetazoline (0.70 mM) and methoxamine (0.74 mM). Tramazoline, methoxamine and oxymetazoline did not induce a full aggregation of melanosomes even at the maximal concentration examined (Fig. 9). The other agonists showed full melanosome-aggregating action, indicating that they are all full agonists.

Effects of antagonists

Effects of antagonists, prazosin, corynanthine, yohimbine and phentolamine were examined on norepinephrine- or clonidine-induced melanosome-aggregated melan-

ophores. In these experiments, concentrations of the agonists used were 50 nM for norepinephrine and 1 μM for clonidine, respectively, which induced a full melanosome aggregation of melanophores (cf. Fig. 9). Melanophores were pretreated with a given concentration of each antagonist for 5 min, and then were treated with either agonist in the presence of a respective antagonist.

Figure 10 shows typical examples for the effects of prazosin and yohimbine on norepinephrine-treated melanophores. Both antagonists inhibited melanosome-aggregation response of the cells to 50 nM norepinephrine in a concentration-dependent manner. A full inhibition was obtained with prazosin at 10 μM and with yohimbine at 0.1 μM, respectively. These antagonists also inhibited the cell response to 1 μM clonidine in a similar fashion (Fig. 11). Although the data are not shown here, prazosin and yohimbine inhibited cell responses to the other alpha agonists used, in which the inhibitory effect of yohimbine was always observed to be about 100-fold greater than that of prazosin.

Figure 12 presents the relation between concentrations of antagonists and their inhibitory effects on melanosome-aggregation response of melanophores to 50 nM norepinephrine (a) and 1 μM clonidine (b). In the figure, effect of oxymetazoline which itself is known as an alpha agonist is also shown. The drug had no

melanosome-aggregating action at concentrations lower than 1 μM (cf. Fig. 9), but it inhibited the melanophore responses to norepinephrine and clonidine just as being an antagonist. The order of the inhibitory effect of antagonists on the cell response to norepinephrine was observed to be the same as that of the drugs on the cell response to clonidine. The effective concentrations of antagonists for inducing 50% inhibition of cell response to 50 nM norepinephrine were 14.5 nM, 85.8 nM, 0.84 μM, 1.3 μM, and 11.7 μM for yohimbine, phentolamine, oxymetazoline, prazosin and corynanthine, and those to 1 μM clonidine were 14.0 nM, 0.54 μM, 1.6 μM, 1.8 μM, and 24.5 μM, respectively.

Discussion

At present, numerous evidence have revealed that the adrenergic receptors mediating melanosome aggregation within melanophore of teleost fishes are alpha in nature (cf. Bagnara and Hadley, 1973; Fujii and Oshima, 1986). In the present experiments on medaka melanophores, melanosome-aggregating potency of clonidine, naphazoline and tramazoline, all known as alpha-2 agonists, were far greater than that of alpha-1 agonists, i.e. methoxamine, phenylephrine and tetrahydrozoline. Furthermore, the inhibitory action of yohimbine, a specific alpha-2 antagonist, on melanophore response to norepinephrine was about 10-fold greater than that of phentolamine, a nonspecific antagonist, and was about 100-fold greater than that of prazosin and corynanthine, specific alpha-1 antagonists, respectively. Such differences among the inhibitory actions of antagonists were observed to become far greater when the antagonist effects were examined on the cell response to clonidine. Critical estimation of the effects of these agents, based on their proven actions in other animals, demonstrates that the subtype of alpha adrenergic receptors involved in the melanosome-aggregation response of medaka melanophores is probably of alpha-2.

Subclassification of alpha adrenergic receptors have first been made on mammalian tissues, in which

Langer (1974) proposed that postsynaptic receptors be referred to as alpha-1 and the presynaptic receptors as alpha-2. However, subsequent reseraches have demonstrated that the alpha-2 receptors also exist in the postsynaptic tissues, and the terms alpha-1 and alpha-2 receptors are now being used solely for receptors with different reltive activities and affinities of agonists and antagonists, respectively, irrespective of the location (Berthelsen and Pettinger, 1977; Hoffman et al., 1079; Timmermans and van Zwieten, 1982). In the present study, since melanophores used were exclusively denervated ones, alpha receptors involved in the melanosome-aggregation response of the present cells are postsynaptic ones.

In a recent study on scale melanophores of the teleost, Labrus ossifagus, Andersson et al. (1984) have determined that adrenergic receptors regulating melanosome aggregation are alpha-2 in nature, demonstrating that yohimbine inhibits melanophore response to norepinephrine completely but prazosin practically does not. Carter and Shuster (1982) have obtained a similar result on skin melanophores of the lizard, Anois carolinensis, indicating that the receptors involved in melanosome-aggregation response of the cells are also alpha-2. However, in the present experiments, high concentrations of alpha-1 antagonists inhibited the melanophore response to norepinephrine and clonidine,

respectively, and besides, alpha-1 agonists could also induce melanosome aggregation within the cells, though the potencies were considerably low. Furthermore, melanosome-aggregating effects of norepinephrine and epinephrine, nonspecific agonists, were observed to be about 10-fold greater than that of specific alpha-2 agonists. These findings suggest that the alpha receptor population of the present melanophores consists of both alpha-1 and alpha-2 subtypes. Coexistence of alpha-1 receptors with alpha-2 receptors has been demonstrated in rabbit uterus cells (Hoffman et al., 1979) and rat hepatocytes (Hoffman et al., 1980). Ruffolo and Waddell (1982) have observed that alpha receptor of rat aorta has properties of both alpha-1 and alpha-2 receptors. However, because of the observed difference in the affinities of melanophores to specific agonists and antagonists, the present results indicate that alpha-2 receptors play a major role in melanosome-aggregation response of the medaka melanophores.

Among alpha agonists used in the present experiments, the action of oxymetazoline was observed to be considerably different from that demonstrated in other tissues. Oxymetazoline and clonidine, both are imidazoline derivatives, act as alpha-2 agonists on presynaptic alpha receptors of rabbit plumonary artery (Starke et al., 1975) and on presynaptic alpha receptors of guinea pig ileum (Wikber, 1978). In rat brains,

alpha-2 action of oxymetazoline is observed to be far greater than that of clonidine (De Jong and Soudijn, 1981). Meanwhile, Lasch and Jakobs (1979) have demonstrated in human platelets that, though epinephrine inhibits adenylate cyclase activity through stimulation of alpha-2 receptor, both oxymetazoline and clonidine act as antagonists, reducing the epinephrine-induced inhibition of enzyme. In the present expeiment, clonidine acted as a potent alpha-2 agonist on melanophore, but oxymetazoline was far inferior in melanosome-aggregating action and besides it acted rather as an antagonist on the cells, inhibiting the melanosome-aggregating action of clonidine. All these findings suggest that the nature of alpha-2 receptor differs considerably among the cells examined and that oxymetazoline acts as an agonist on the presynaptic alpha-2 receptors, while it acts on postsynaptic alpha-2 receptors as an antagonist.

Another imidazoline derivative, tetrahydrozoline, is shown to act as an antagonist on alpha-2 receptors of human platelets (Lasch and Jakobs, 1979). However, Ruffolo and Waddell (1982) have demonstrated that tetrahydrozoline has no alpha-2-agonist activity in rat aorta, while it shows an alpha-1 agonistic action in rabbit aorta. In contrast to these, De Jong and Soudijn (1981) in human platelets and Timmermans and van Zwieten (1981) in rat vascular smooth muscles have

demonstrated that tetrahydrozoline acts as alpha-2 agonist. In the present experiments, malanosome-aggregating action of tetrahydrozoline was approximately the same as other alpha-2 agonists, such as clonidine, naphazoline and tramazoline. All the above findings suggest that the difference in the action of tetrahydrozoline on different animal cells is due to the difference in the molecular structure of receptors in the corresponding cells. As there is no available evidence at present, further invistigations is necessary.

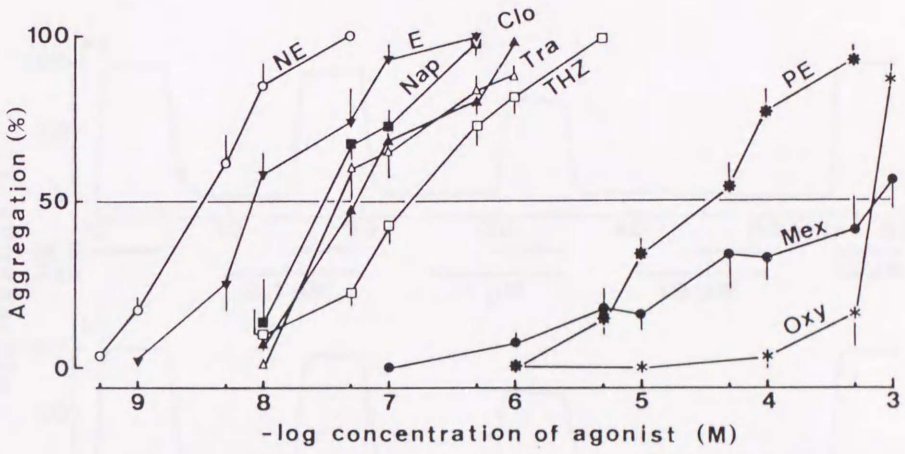

Fig. 9. Relationship of the concentration of adrenergic alpha agonists to the magnitude of melanosome-aggregation response of the medaka melanophores. Abscissa, concentration of agonists in logarithmic scale. Ordinate, magnitude of aggregation response (%). Each point is the mean of 8 measurements in different animals and vertical bars indicate SEs. Clo. clonidine; E, epinephrine; Mex, methoxamine; Nap, naphazoline; NE, norepinephrine; Oxy, oxymetazoline; PE. phenylephrine; THZ, tetrahydrozoline; Tra, tramazoline.

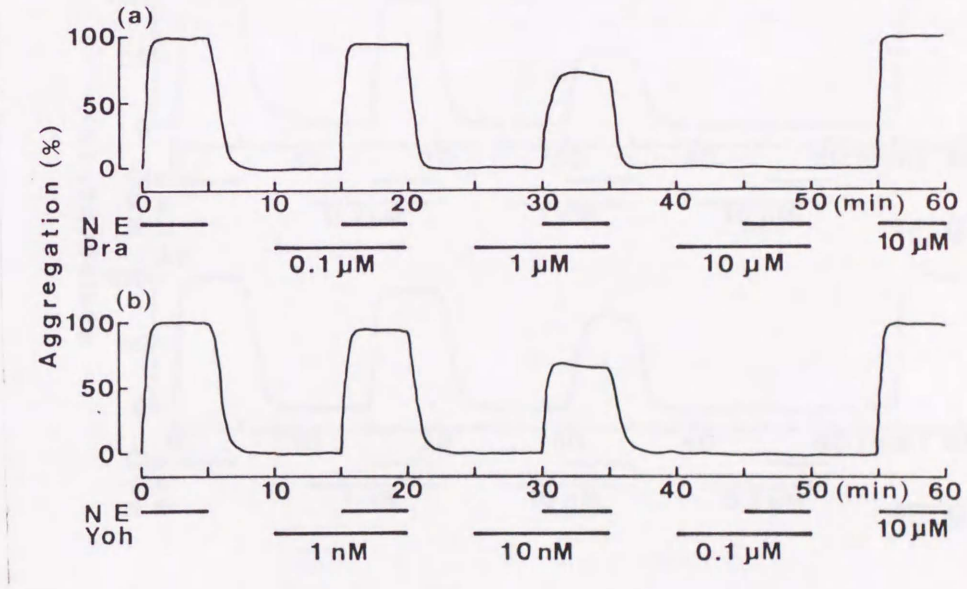

Fig. 10. Typical recordings showing the inhibitory effects of (a) prazosin (Pra) and (b) yohimbine (Yoh) on melanosome-aggregation response of the medaka melanophores to 50 nM norepinephrine (NE). Abscissae, time in min. Ordinates, magnitude of aggregation response (%).

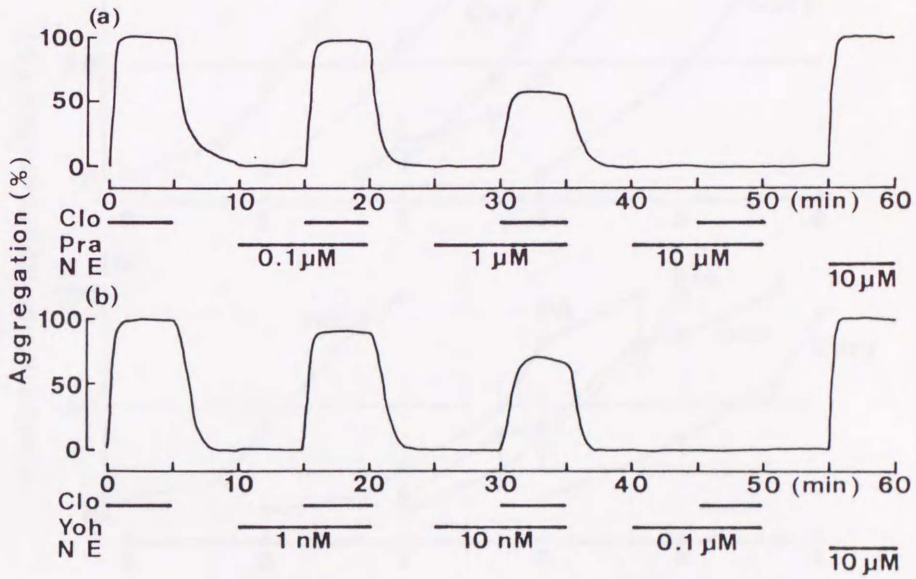

Fig. 11. Typical recordings showing the inhibitory effects of (a) prazosin and (b) yohimbine (Yoh) on melanosome-aggregation response of the medaka melanophores to 1 μM clonidine (Clo). Abscissae, time. Ordinates, magnitude of aggregation response (%).

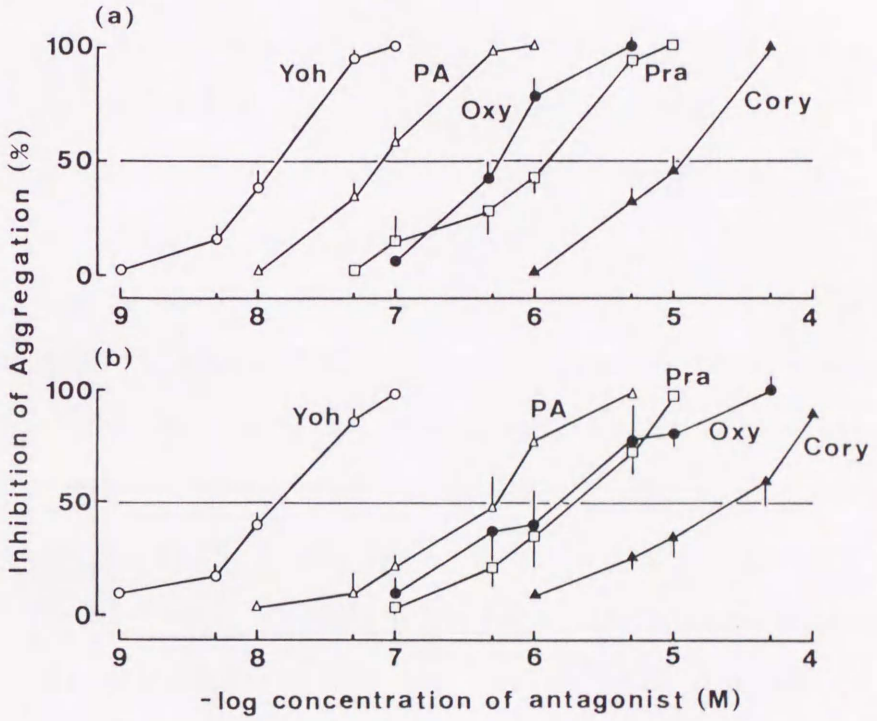

Fig. 12. Inhibition of (a) norepinephrine (50 nM NE)-induced and (b) clonidine (1 μM Clo)-induced melanosome-aggregation response of the medaka melanophores by various adrenergic alpha antagonists. Abscissae, concentrations of antagonists in logarithmic scale. Ordinates, inhibition of aggregation (%). Each point is the mean of 8 measurements in different animals and vertical bars indicate SEs. Cory, corynanthine; Oxy, oxymetazoline; PA, phentolamine; Pra, prazosin; Yoh, yohimbine.

V. The subtype of alpha adrenergic receptors mediating leucosome aggregation in leucophores

In scales of the medaka, leucophores are usually found to be located just beneath melanophores. Nerve-mediated stimuli, such as electric currents and potassium ions, and exogenous norepinephrine induce aggregation of melanosomes within melanophores, while at the same time, the stimuli cause dispersion of leucosomes within leucophores (Miyoshi, 1952; Iga, 1969, 1978). Notwithstanding such difference in the response to identical stimuli, it has been suggested that melanophores and leucophores receive the same sympathetic postganglionic fibers (Iwata et al., 1981; Iga 1983; Yamada et al., 1984).

On the other hand, pharmacological investigations on medaka chromatophores have revealed that adrenergic receptors which mediate aggregation of pigment granules within melanophores and lecuophores are alpha receptors (Iga, 1968; 1979) and that those which madiate dispersion of pigment granules within these chromatophores are beta in nature (Obika, 1976; Iga et al., 1977; Yamada, 1980; Komatsu and Yamada, 1982).

In the foregoing studies (Chapter III and IV), according to the criteria for subclassification of adrenergic receptors in mammalian tissues (Lands et al., 1967; Minneman et al., 1979; Starke, 1981; Johansson, 1984), I had examined the subtypes of adrenergic receptors in medaka chromatophores and determined that beta adrenergic receptors madiating dispersion of

pigment granules in melanophores and leucophores are beta-2 and beta-1, respectively, and that the alpha adrenergic receptors which mediate melanosome aggregation in melanophores are alpha-2 in the subtype.

These findings suggest that the observed difference in response to nerve stimuli and exogenous norepinephrine between malanophores and leucophores is due to differences in the subtypes of adrenergic receptors and in the affinity of receptors to neurotransmitter, norepinephrine. However, at present, the subtype of alpha adrenergic receptors, which mediate leucosome aggregation in leucophores has not yet been determined. Therefore, in the present study, using specific agonists and antagonists, subclassification of alpha receptors in medaka leucophores was undertaken as an aid for further analysis of the receptor mechanisms involved in the regulation of chromatophore responses.

Results

Effects of catecholamines

First, leucosome-aggregating effects of representative catecholamines, i.e. norepinephrine, epinephrine and isoproterenol, on leucophores were investigated. Since leucophores usually maintain a fully leucosome-aggregated state in physiological saline, effects of the amines were examined on leucosome-dispersed cells pretreated with alpha-MSH. As shown in Figure 13, 0.1 nM MSH induced about 90% dispersal of leucosomes.

Among the catecholamines examined, norepinephrine and isoproterenol caused a further increase in the degree of cell responses to MSH (Fig. 13a and b). These effects of amines were observed to be concentration-dependent (data not shown). In the presence of propranolol (5 μM), a beta antagonist, however, the effects of amines were completely reversed and leucophores responded with aggregation of leucosomes (Fig. 13a and b). The aggregating effects of amines were also concentration-dependent and leucophores responded with full pigment aggregation to 0.5 nM norepinephrine and 2 μM isoproterenol, respectively (Figs. 13a, b and 14). When phentolamine (5 μM), an alpha antagonist, was further added, such aggregation response of leucophores to the amines were markedly inhibited and the cells soon reached to a leucosome-dispersed state (Fig.

13a and b).

Meanwhile, epinephrine, another catecholamine, induced aggregation of leucosomes in MSH-treated dispersed leucophores even in the absence of propranolol (Fig. 13c). The effective concentration of the amine was more than 1 nM and the full leucosome aggregation was obtained at 20 nM (Fig. 14). However, at concentrations higher than 50 nM, the aggregating effect of epinephrine decreased with increase in the concentration and the cells assumed a leucosome dispersed state, attaining a fully dispersed state at more than 0.5 μM (Figs. 13c and 14).

Simultaneous application of propranolol completely abolished the dispersion response of leucophores to high concentrations of epinephrine (Figs 13c and 14), but it had no influence on the leucosome-aggregating effect of the low concentrations of amine (Fig. 14). The aggregation response of leucophores to epinephrine was also inhibited by the presence of phentolamine (Fig. 13c).

The leucosome-aggregating potency of catecholamines and the EC_{50}s estimated from Fig. 14 were in the following order: epinephrine (2.1 nM), norepinephrine (27.0 nM) and isoproterenol (180.0 nM).

Effects of specific alpha agonists

Effects of various concentration of prazosin, an

alpha-1 antagonist, and yohimbine, an alpha-2 antagonist, were examined. Similar to the foregoing experiment, leucophores were pretreated with 0.1 nM MSH in the presence of 5 μM propranolol, during which the effect of each antagonist was examined on the cell response to 0.5 μM norepinephrine.

Figure 15 shows typical recordings of the effects of yohimbine and prazosin. Both antagonists inhibited the aggregation response of leucophores to norepinephrine in a concentration-dependent manner. As evident from the figure, the effect of yohimbine was about 100-fold greater than that of prazosin. Although the data are not shown, similar inhibitory effects of antagonists were observed on the cell responses to isoproterenol and epinephrine, respectively.

The relation between concentrations and the inhibitory effects of antagonists on the cell response to norepinephrine is shown in Figure 16. The inhibitory potency and the effective concentration of antagonists for inducing 50% inhibition of the cell response estimated from the figure were in the following order: yohimbine (28.2 nM), phentolamine (0.49 μM) and prazosin (4.2 μM).

Effects of specific alpha agonists

Effects of alpha-1 agonists, methoxamine and phenylephrine, and alpha-2 agonists, clonidine and oxy-

metazoline, were examined on MSH-induced leucosome-dispersed leucophores. Figure 17 shows typical recordings of the effects of methoxamine and clonidine. As is apparent from the figure, leucosome-aggregating potency of the agonists was practically unchanged in the presence of propranolol.

Figure 18 shows the relationship of the magnitude of leucosome-aggregation response of leucophores to the concentrations of agonists. The leucosome-aggregating potency of agonists and the EC_{50}'s estimated from the curves were in the following order: clonidine (0.4 μM), oxymetazoline (0.59 μM), methoxamine (2.2 μM) and phenylephrine (3.3 μM).

Discussion

Leucophores in scales of the medaka usually maintain a fully leucosome-aggregated state in physiological saline. Heretofore physiological and pharmacological investigations have demonstrated that catecholamines induce dispersion of leucosomes in the cells through mediation of beta adrenergic receptors (Obika, 1976; Iga et al., 1977; Yamada, 1980), while MSH causes leucosome dispersion via activation of MSH-receptors (Negishi and Obika, 1980; Yamada, 1982; Oshima and Fujii, 1985). Besides, it has been shown that exogenous cyclic AMP, methylxanthines (inhibitors of the cyclic nucleotide phosphodiesterase) and forskolin (an activator of adenylate cyclase) also induce dispersion of leucosomes (Obika, 1976; Negishi and Obika, 1980; Yamada and Iwakiri, 1982; Namoto and Yamada, 1987).

Working on theophylline-treated leucosome-dispersed medaka leucophores, Iga (1979) has observed that under the influence of propranolol catecholamines produce aggregation of leucosomes and that this catecholamine effect is completely abolished by further addition of dibenamine, an adrenergic alpha blocker. He concluded from the results that alpha adrenergic receptors which mediate leucosome aggregation are present in medaka leucophores, in addition to beta adrenergic receptors mediating leucosome dispersion. In the present

experiment, to maintain leucophores in a leucosome-dispersed state, the cells were pretreated with MSH instead of theophylline. In such cells, catecholamines also induced aggregation of leucosomes in the presence of propranolol, and the effects of amines were markedly inhibited by the coexistence of phentolamine. The order of leucosome-aggregating potency of catecholamines obtained in the present study well coincided with that has been observed by Iga (1979). Thus, the present findings also strongly substantiated the presence of alpha adrenergic receptors in medaka leucophores, which are associated with aggregation of leucosomes.

The present results revealed further that both prazosin, an alpha-1 antagonist, and yohimbine, an alpha-2 antagonist, competitively inhibited the leucosome-aggregating actions of catecholamines on MSH-dispersed leucophores and that the inhibitory potency of yohimbine was about 100-fold greater than that of prazosin. Besides, both alpha-1 agonists, methoxamine and phenylephrine, and alpha-2 agonists, clonidine and oxymetazoline, induced leucosome aggregation in MSH-dispersed leucophores regardless of the presence or absence of propranolol. However, leucosome-aggregating potency of alpha-2 agonists was observed to be about 10-fold greater than that of alpha-1 agonists. These results clearly indicate that the subtype of alpha adrenergic receptors which mediate leucosome-aggregation

response of medaka leucophores is alpha-2.

As described in Chapter IV, the subtype of alpha adrenergic receptors mediating melanosome aggregation within medaka melanophores are also alpha-2. However, the affinity of the receptors to agonists is somewhat different between leucophores and melanophores. For melanophores, the order in potency of catecholamines to induce melanosome aggregation is: norepinephrine, epinephrine and isoproterenol, in contrast to the order that obtained for leucophores, i.e. epinephrine, norepinephrine and isoproterenol. In addition, oxymetazoline acted as a potent alpha agonist on leucophores, while the agent itself had no effect on melanophores and rather acted as an antagoists, inhibiting the cell responses to catecholamine. Such a difference in the action of oxymetazoline on alpha-2 adrenergic receptors has been shown in various other tissues. Oxymetazoline acts as an alpha-2 agonist on presynaptic alpha receptors of both rabbit pulmonary artery (Starke et al., 1975) and guinea pig ileum (Wikberg, 1978), and on rat brains (De Jong and Soudijn, 1981), while it acts as an alpha-2 antagonist on human platelets (Lasch and Jakobs, 1979). All these findings suggest that the nature of alpha-2 receptors differs considerably among the cells examined.

In the present study, epinephrine, at low concentrations (less than 50 nM), itself could induce aggre-

gation of leucosomes in MSH-dispersed leucophores even in the absence of propranolol, while it caused a leucosome dispersion at higher concentrations (more than 50 nM). Since epinephrine is generally known to act as both alpha and beta agonists on adrenergic tissues, it may be said that, at lower concentrations, the amine acted on alpha receptors and induced aggregation of leucosomes within leucophores, while at higher concentrations, it acted on beta receptors and caused a dispersion of leucosomes overcoming the alpha receptor-mediated action.

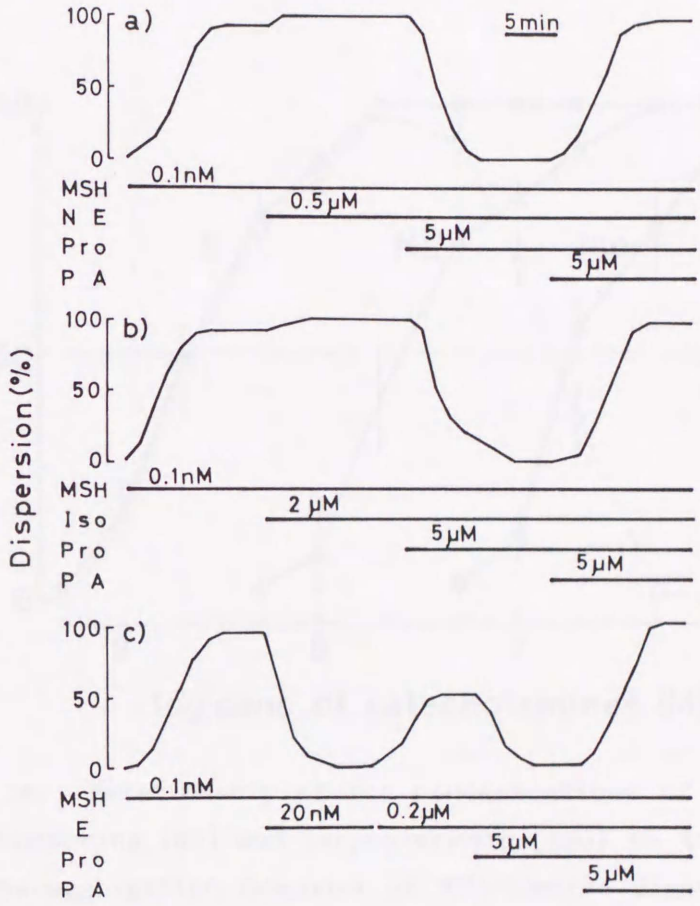

Fig. 13. Typical recordings showing the effects of (a) norepinephrine (NE), (b) isoproterenol (Iso) and (c) epinephrine (E) on the leucosome-dispersed medaka leucophores treated with 0.1 nM MSH, before and during application of 5 μM propranolol (Pro) and 5 μM phentolamine (PA). Abscissae, time in min. Ordinates, degree of leucosome dispersion.

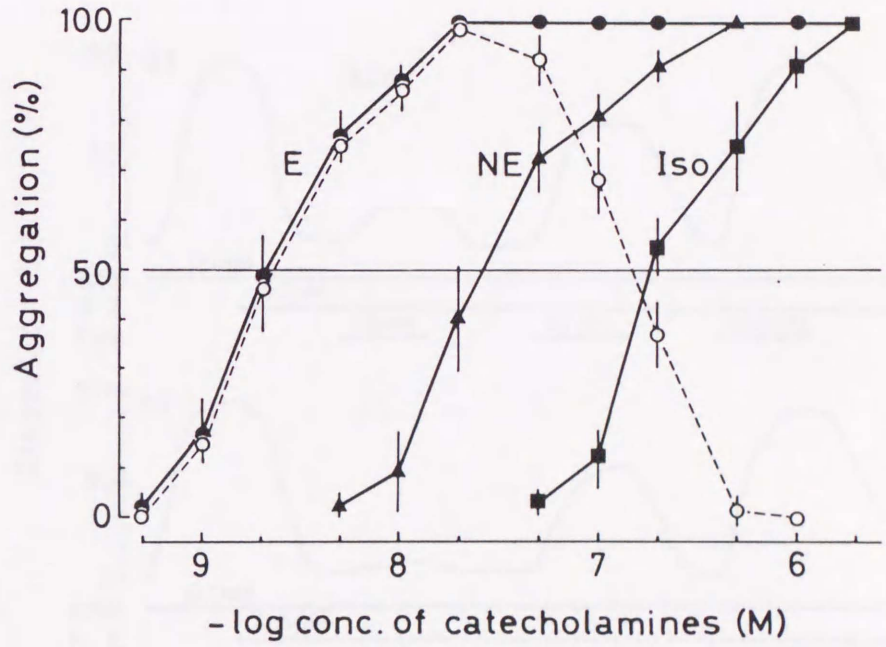

Fig. 14. Relationship of the concentrations of epinephrine (E), norepinephrine (NE) and isoproterenol (Iso) to the magnitude of leucosome-aggregation response of MSH-treated dispersed leucophores in the presence (solid line) and absence (broken line for E) of 5 μM propranolol. Abscissa, concentration of catecholamines in logarithmic scale. Ordinate, magnitude of aggregation response (%). Each point is the mean of 20 measurements in different animals and vertical bars indicat SEs.

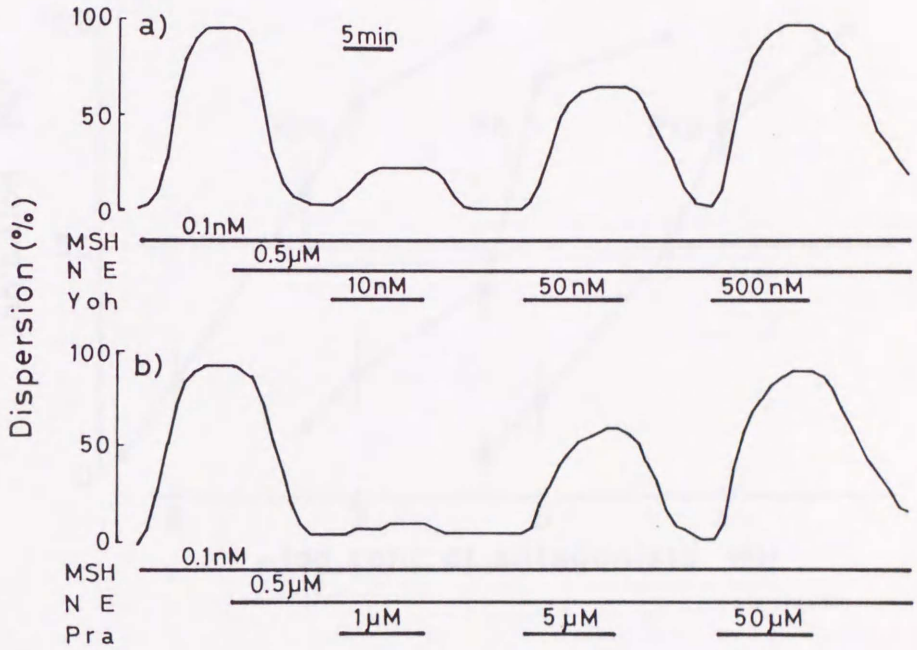

Fig. 15. Typical recordings showing the inhibitory effects of (a) yohimbine (Yoh) and (b) prazosin (Pra) on leucosome-aggregation response of MSH-treated dispersed leucophores to 0.5 μM norepinephrine (NE) in the presence of 5 μM propranolol (although not indicated in figure, propranolol was applied simultaneously with MSH). Abscissae, time in min. Ordinates, degree of leucosome dispersion (%).

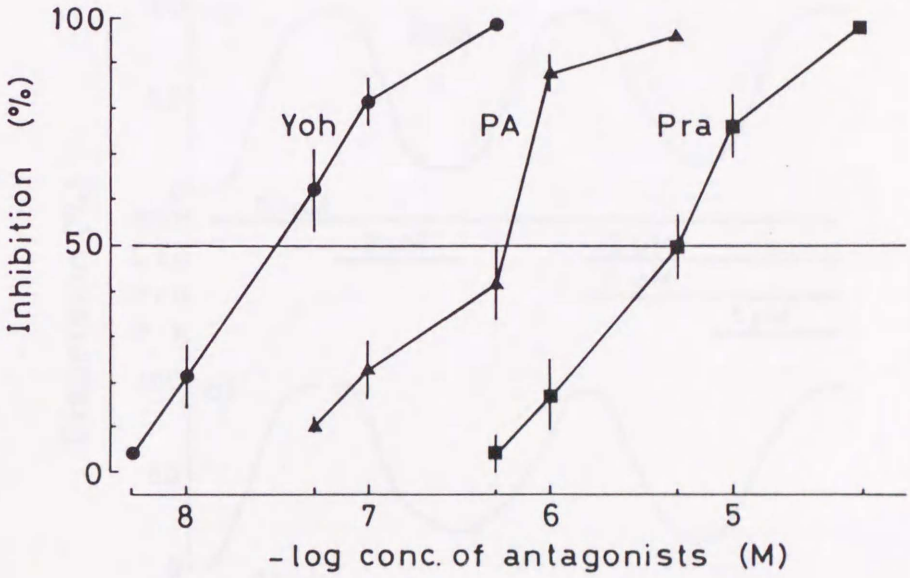

Fig. 16. Relation between the concentration and the degree of inhibitory effects of alpha antagonists, yohimbine (Yoh), phentolamine (PA) and prazosin (Pra) on leucosome-aggregation response of MSH-treated dispersed leucophores to 0.5 μM norepinephrine in the presence of 5 μM propranolol. Abscissa, concentration of antagonists in logarithmic scale. Ordinate, degree of inhibition (%). Each point is the mean of 20 measurements in different animals and vertical bars indicate SEs.

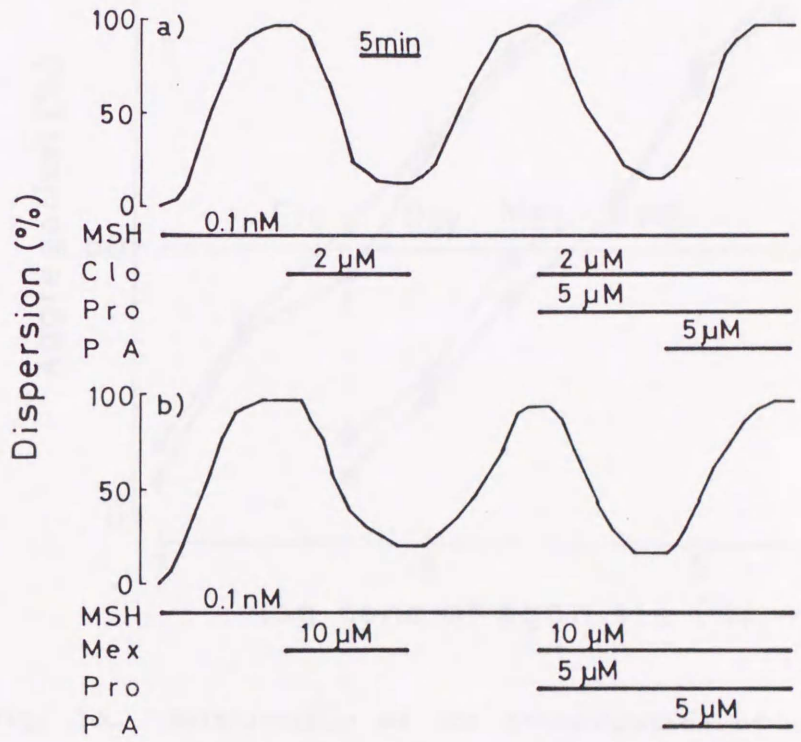

Fig. 17. Typical recordings showing the effects of (a) clonidine and (b) methoxamine (Mex) on MSH-treated dispersed leucophores before and during application of 5 µM propranolol (Pro) and 5 µM phentolamine (PA). Abscissae, time in min. Ordinates, degree of leucosome dispersion (%).

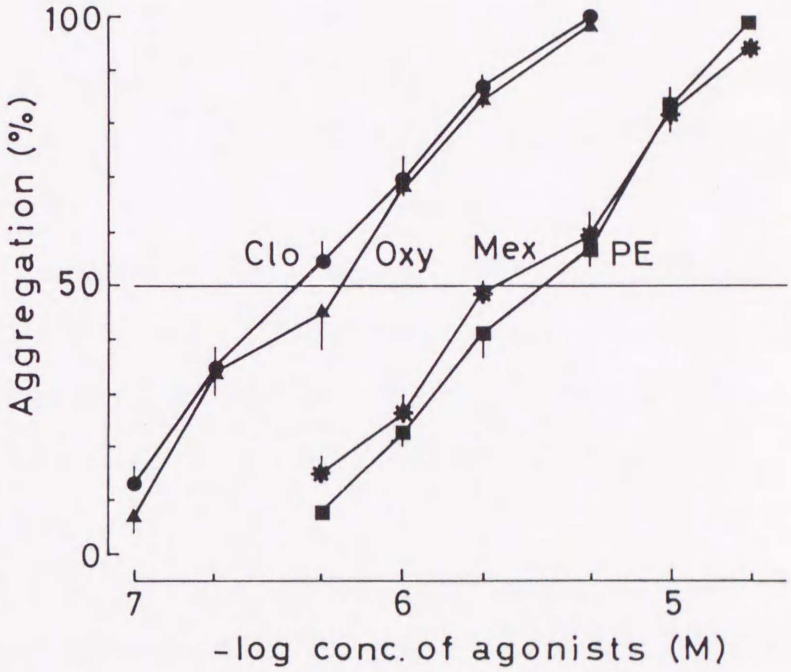

Fig. 18. Relationship of the concentration of alpha agonists, clonidine (Clo), oxymetazoline (Oxy), methoxamine (Mex) and phenylephrine (PE) to the magnitude of leucosome-aggregation response of MSH-treated dispersed leucophores. Abscissa, concentration of agonists in logarithmic scale. Ordinate, magnitude of aggregation response (%). Each point is the mean of 10 measurements in different animals and vertical bars indicate SEs.

VI. General discussion

In the wild type of the medaka, both melanophores and leucophores are present in scales and are involved in the control of physiological color changes. Recent investigations have suggested that melanophores and leucophores of the medaka receive the same adrenergic innervation and that the neurotransmitter released is norepinephrine (Iwata et al., 1981; Iga, 1983; Yamada et al., 1984). Besides, pharmacological researches have demonstrated that these two types of chromatophores have both alpha and beta adrenergic receptors (Iga, 1968; Obika, 1976; Iga et al., 1977; Yamada, 1980). However, notwithstanding these findings, usually nerve-mediated stimuli or exogenous norepinephrine induce aggregation of melanosomes within melanophores and at the same time dispersion of leucosomes within leucophores.

In the present study, to analyse the receptor mechanisms involved in the regulation of chromatophore responses more precisely, the subtypes of adrenergic receptors in medaka chromatophores were examined according to the criteria for subclassification of adrenergic receptors in mammals. The results demonstrated that the beta receptors which mediate dispersion of pigment granules within leucophores and melanophores are beta-1 and beta-2, respectively (Chapter III), and that the alpha receptors mediating aggregation of pigment granules within these two chromatophores are both

alpha-2 in nature (Chapters IV and V). However, affinities of the alpha-2 receptors for catecholamines were observed to differ considerably between melanophores and leucophores, i.e. the receptors in melanophores are more sensitive to norepinephrine than epinephrine, but the reverse is the case for those in leucophores.

In mammalian adrenergic tissues, norepinephrine is generally accepted to be beta-1 agonist as well as alpha agonist (Lands et al., 1967; Carlsson and Hedberg, 1976; Minneman and Molinoff, 1980). Since the beta adrenergic receptors in medaka melanophores are not beta-1 but are beta-2 in subtype, the neurotransmitter or exogenous norepinephrine does not act on them, but acts selectively on alpha-2 receptors to induce aggregation of melanosomes. On the other hand, the subtypes of beta receptors in leucophores are exclusively beta-1 and the alpha receptors responsible for leucosome aggregation are alpha-2 in nature. However, as mentioned above, the affinity of alpha-2 receptors of leucophores for norepinephrine is comparatively low so that the cells properly respond to neurotransmitter or exogenous norepinephrine with dispersion of leucosomes through mediation of beta-1 receptors. Then, it can be concluded that through such a difference in the receptor subtypes, melanophores and leucophores behave in directions quite opposite to each other.

Miyashita and Fujii (1975) have demonstrated that melanophores of the guppy possess both alpha and beta adrenergic receptors and suggested that the endogenous beta amine responsible for dispersion of melanosomes is epinephrine and that its source is chromaffin cells in adrenal tissues or in the dermis. The same may also be applied to the present melanophores, since the subtype of beta receptors in the cells is beta-2 and epinephrine but not norepinephrine had a melanosome-dispersing potency. With respect to leucophores, epinephrine, but not norepinephrine could induce an aggregation of leucosomes in the MSH-treated dispersed cells even in the absence of beta antagonist (Chapter V). Since norepinephrine is the neurotransmitter of the adrenergic nerves innervating medaka chromatophores, it can be said that, in vivo, the neurotransmitter solely induces melanosome aggregation of melanophores through mediation of the alpha-2 receptors and at the same time dispersion of leucosomes within leucophores via activation of the beta-1 receptors. Meanwhile, endogenous epinephrine may cause melanosome dispersion of melanophores through mediation of the beta-2 receptors and aggregation of leucosomes within leucophore acting on the alpha-2 receptors.

In various adrenergic tissues of mammals, stimulation of beta adrenergic receptors has been shown to activate adenylate cyclase and induce proper functions

of cells through increase in the intracellular level of cyclic AMP (Robison et al., 1967; Minneman et al., 1979; Cerione et al., 1983, 1984), while stimulation of alpha receptors, especially alpha-2 receptors, inhibits the enzyme activity vice versa (Lasch and Jakobs, 1979; Burns et al., 1982).

In fish species, exogenous cyclic AMP has been demonstrated to induce a dispersion of melanosomes within epinephrine-treated aggregated melanophores of Fundulus (Novales and Fujii, 1970), the goldfish, Carassius auratus (Abramowitz and Chavin, 1974), the guppy (Fujii and Miyashita, 1976) and the medaka (Negishi and Obika, 1980). Furthermore, using radio-immunoassay methods, Negishi et al. (1981, 1982) have demonstrated in melanoma cells of a hybrid fish that the intracellular cyclic AMP content of epinephrine-aggregated cells is significantly less than that of non-treated cells and that theophylline, which induces dispersion of melanosomes, causes a remarkable increase in cellular cyclic AMP. Andersson et al. (1984) have also observed that norepinephrine induces a significant reduction of cyclic AMP content in Labrus melanophores. Namoto and Yamada (1983) and Namoto (1985) have shown that Li^{+}, known as an inhibitor of adenylate cyclase (Duosa and Hetcher, 1970; Wang et al., 1974; Ebstein et al., 1976), induces melanosome aggregation of medaka melanophores and that Mg^{2+} and Mn^{2+}, known as acceler-

ators of the enzyme (Drummond and Duncan, 1970; Halmi et al., 1974), produce inhibition of melanosome-aggregation response to norepinephrine or Li^+. Forskolin, a potentiator of adenylate cyclase (Metzger and Linder, 1981; Seamon et al., 1981; Sano et al., 1983), has also been shown to induce a dispersion of melanosome within norepinephrine-treated aggregated Labrus melanophores (Andersson et al., 1984) and in Li^+-treated medaka melanophores (Namoto, 1987). In leucophores of the medaka, it has been also observed that both exogenous cyclic AMP and methylxanthines, such as caffeine and theophylline (Yamada and Iwakiri, 1982), and forskolin (Namoto and Yamada, 1987) cause a dispersion of leucosomes, while Li^+ inhibits leucosome-dispersion response of the cells to isoproterenol (Namoto and Yamada, 1987). All these findings together with the present results suggest that stimulation of alpha-2 adrenergic receptors in fish melanophores inhibits adenylate cyclase activity and causes a melanosome-aggregation response through reduction of cellular cyclic AMP content, while stimulation of beta-2 receptors in leucophores activates the enzyme and induces dispersion of leucosomes by elevating the intracellular level of cyclic AMP.

Recent investigations on mammalian tissues have revealed that in hormone- and neurotransmitter-sensitive adenylate cyclase systems, guanine nucleotide-

binding regulatory proteins (G-proteins) play an important role in the communication between receptors and adenylate cyclase (cf. Rodbell, 1980; Gilman, 1984). In beta adrenergic receptor-mediated systems the stimulatory G-protein (Gs) is involved and the binding of agonist with the receptors activates Gs, which stimulates the adenylate cyclase activity, while in alpha-2 adrenergic receptor-mediated responses, the inhibitory G-protein (Gi) is involved, stimulation of which mediates an inhibition of the enzyme. From the subtype analogy of adrenergic receptors between medaka chromatophores and mammalian tissues, it is tentatively assumed that the adrenergic regulation of pigment movements in medaka chromatophores may be mediated by adenylate cyclase system including these two GTP-binding proteins, Gs and Gi. Cholera toxin has been shown to catalyze the ADP-ribosylation of Gs and to augument the adenylate cyclase activity (Cassel and Pfeuffer, 1978; Gill and Meren, 1978), while islet-activating protein (IAP), one of the pertussis toxins, is known to block the receptor-mediated signal transduction through inhibition of the adenylate cyclase activity by the ADP-ribosylation of Gi (Katada and Ui, 1981, 1982). Recently, Karlsson et al. (1985) have demonstrated that alpha-2 receptor-mediated melanosome-aggregation response of Labrus melanophores is markedly inhibited by the presence of IAP and have suggested the

involvement of Gi in the signal transduction system. However, the involvement of GTP-binding proteins, especially that of Gs, in the receptor-mediated regulation of adenylate cyclase in fish chromatophores still remaine to be studied.

VII. Summary

Melanophores and leucophores in scales of the medaka, Oryzias latipes (wild type), have been known to receive the same sympathetic postganglionic fibers and the released neurotransmitter has been suggested to be norepinephrine. Moreover, these two types of chromatophores have been shown to possess both alpha adrenergic receptors which mediate aggregation of pigment granules and beta receptors which mediate pigment dispersion. However, nervous stimuli or exogenous norepinephrine usually induce an opposite response of the cells to each other, i.e. aggregation of melanosomes within melanophores and dispersion of leucosomes within leucophores.

In the present study, to analyse the receptor mechanisms involved in the regulation of such opposite responses of medaka chromatophores, subtypes of adrenergic receptors in the cells were determined according to the criteria for subclassification of the receptors in mammalian tissues, using representative catecholamines and specific adrenergic agonists and antagonists.

In the first series of experiments (Chapter III), subtypes of beta adrenergic receptors responsible for both melanosome dispersion of melanophores and leucosome dispersion of leucophores were examined.

Both norepinephrine, epinephrine and isoproterenol induced an aggregation of melanosomes within melano-

phores and propranolol, a beta antagonist, augmented the action of the amines except that of norepinephrine. This suggest that both epinephrine and isoproterenol have a melanosome-dispersing action on melanophores in addition to the melanosome-aggregating action and that propranolol augments the aggregation response of the cells to the amines through inhibition of beta adrenergic receptors. Salbutamol and terbutaline, specific beta-2 agonists, competitively inhibited the melanosome-aggregation response of melanophores to norepinephrine, while the specific beta-1 antagonists, metoprolol and atenolol, did not interfere with the melanosome-dispersion response of the cells to isoproterenol. These findings indicate that the subtype of beta adrenergic receptors which mediate the dispersion of melanosomes within melanophores is beta-2.

With regard to leucophores, all the catecholamines examined produced a dispersion of leucosomes within the cells. Meanwhile, beta-2 agonists, salbutamol and terbutaline which are also known to be alpha-1 antagonists, had no leucosome-dispersing effect on the cells, but inhibited the leucosome-dispersion response of the cells to isoproterenol, and metoprolol and atenolol, beta-1 antagonists, also competitively inhibited the leucophore response to isoproterenol, indicating that the beta adrenergic receptors mediating leucosome dispersion of leucophores are beta-1 in subtype.

In the next series of experiments (Chapter IV), the subtype of alpha adrenergic receptors which mediate the melanosome-aggregation response of melanophores was examined. Both specific alpha-1 agonists, methoxamine, phenylephrine and tetrahydrozoline, and specific alpha-2 agonists, clonidine, naphazoline and tramazoline, induced an aggregation of melanosomes in the cells. However, the melanosome-aggregating potency of the alpha-2 agonists was about 10-fold greater than that of the alpha-1 agonists. Among catecholamines examined, norepinephrine was about 5-fold greater in the melanosome-aggregating potency than epinephrine. Furthermore, the inhibitory action of yohimbine, a specific alpha-2 antagonist, on melanosome-aggregation response of melanophores to norepinephrine was about 10-fold greater than that of phentolamine, a non-specific antagonist, and was about 100- and 1000-fold greater than that of prazosine and corynanthine, specific alpha-1 antagonists, respectively. Such differences among the inhibitory actions of antagonists became far greater when the antagonist effects were examined on the cell response to clonidine. All these results indicate that the subtype of alpha adrenergic receptors responsible for the aggregation of melanosomes in medaka melanophores is alpha-2.

In the third series of experiments (Chapter V), the subtype of alpha adrenergic receptors which mediate

the leucosome-aggregation response of leucophores was determined. In these experiments, leucophores were pretreated with MSH (melanophore-stimulating hormone) to maintain the cells in a leucosome-dispersed state. Among the catecholamines examined, norepinephrine and isoproterenol induced an aggregation of leucosomes within the cells in the presence of propranolol, but epinephrine could induce leucosome aggregation even in the absence of the antagonist. Besides, both specific alpha-1 agonists, methoxamine and phenylephrine, and alpha-2 agonists, clonidine and oxymethazoline, also caused leucosome aggregation in leucophores regardless of the presence or absence of propranolol. However, leucosome-aggregating potency of alpha-2 agonists was observed to be about 10-fold greater than that of alpha-1 agonists. Prazosine, a specific alpha-1 antagonist, and yohimbine, a specific beta-2 antagonist, both competitively inhibited the leucosome-aggregation response of the cells to catecholamines, but the inhibitory potency of yohimbine was about 100-fold greater than that of prazosin. These results clearly indicate that the subtype of alpha adrenergic receptors which mediate the leucosome-aggregation response of leucophores is alpha-2.

It was concluded based on the results that since norepinephrine is known to be a beta-1 agonist as well as alpha agonist, the neurotransmitter or exogenous

norepinephrine induced aggregation of melanosomes within the medaka melanophores acting selectively on the alpha-2 receptors but not on beta-2 receptors. On the other hand, the subtypes of beta adrenergic receptors in medaka leucophores were exclusively beta-1 and the alpha adrenergic receptors responsible for leucosome aggregation were alpha-2. However, the affinity of alpha-2 receptors in leucophores for norepinephrine was observed to be comparatively low. Therefore, leucophores could properly respond to neurotransmitter or exogenous norepinephrine with dispersion of leucosomes through mediation of beta-1 receptors. Through such differences in the receptor subtypes, melanophores and leucophores of the medaka could behave in directions quite opposite to each other.

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