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Adrenergic Mechanisms Controlling Pigment Movements in Erythrophores of *Halichoeres tenuispinnis*

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Erythrophores in isolated scales of the motleystripe rainbowfish, *Halichoeres tenuispinnis*, assumed an unstable state in physiological saline solution, changing irregularly their state into a half-aggregated state. The present paper described a method for studying physiological responses of chromatophores that failed to keep a stable state of pigment granules in a physiological saline solution and, using this method, adrenergic regulation of movements of erythrophores in *Halichoeres tenuispinnis* was investigated. The erythrophores maintained the dispersed state in the saline solution for at least 30 min by pretreatment with isoproterenol (10^{-7} M). The treatment seemed not to affect their responses. Norepinephrine-induced aggregation was shown to be mediated by alpha$_2$ adrenoceptors on pharmacological ground. It was also shown that the erythrophores possessed beta adrenoceptors mediating the dispersion of the pigment granules and that the stimulation of the beta adrenoceptors was associated with an increase of the cAMP content within the cells.

Introduction

The skin coloration and its changes in teleost fishes are due to chromatophores and their motility mainly in the dermis of the skin. Fish chromatophores are generally classified into five types according to the colors displayed by them: melanophores (black or brown), erythrophores (red), xanthophores (yellow), leucophores (white) and iridophores (reflecting). Among these chromatophores, it has been assumed that iridophores are non-motile, although in some fish species motile iridophores were reported recently (Lythgoe and Shand, 1982; Oshima *et al.*, 1985; Iga and Matsuno, 1986; Fujii *et al.*, 1989; Iga and Asari, 1991).

The movements of fish chromatophores are generally regulated by sympathetic nerves and hormones. Among these chromatophores, melanophores have been most extensively studied. Recently, however, researches on chromatophores other than melanophores have increased. This may be quite proper from reasons that skin coloration and its color changes are produced in association with various types of chromatophores and that regulation of chromatophorers is diverse in types of chromatophores and also in fish species.

It has been reported that erythrophores of *Myripristis occidentalis* and *Labrus ossifagus* are regulated by alpha$_2$ adrenoceptors (Karlsson *et al.*, 1988; Mårtensson *et al.*,

1990). We know from our experience that, in some fish species, erythrophores in excised preparations fail to keep their state, the pigment dispersion, stable in a physiological saline solution. This was an obstacle to analyze physiological responses of erythrophores. Erythrophores from the motleystripe rainbowfish, which were experimental materials in the present study, also could not keep the state of pigment dispersion in physiological salne solution and assumed irregularly a half-aggregated state.

The purpose of the present investigation was to describe a method for making erythrophores maintain a state of full dispersion and, using this method, to examine adrenergic regulation of movements of erythrophores in the motleystripe rainbowfish, *Halichoeres tenuispinnis*.

Materials and Methods

The motleystripe rainbowfish, *Halichoeres tenuispinnis,* of body length of 9–15 cm were used.

A scale pulled from the fish was attached to a coverslip, which was mounted on a glass trough filled with a physiological saline solution. The composition of the saline solution was as follows: 233 mM NaCl, 8.1 mM KCl, 2.3 mM $CaCl_2$, 3.7 mM $MgCl_2$ and 5.0 mM HEPES-NaOH buffer (pH 7.2).

Responses of erythrophores in the scale were recorded by means of microscopic photographs and changes in diameters of erythrophores were calculated as the degree (%) of an aggregation or a dispersion. In some experiments, changes in diameters of erythrophores were directly measured by means of an ocular micrometer.

The drugs used were as follows: *l*-norepinephrine hydrochloride (Sigma Chemical), *l*-epinephrine bitartrate (Sigma Chemical), clonidine hydrochloride (Sigma Chemical), methoxamine hydrochloride (Sigma Chemical), *l*-phenylephrine hydrochloride (Sigma Chemical), *l*-isoproterenol hydrochloride (Sigma Chemical), yohimbine hydrochloride (Sigma Chemical), corynanthine hydrochloride (Sigma Chemical), *dl*-propranolol hydrochloride (Sigma Chemical), adenosine (Wako Chemicals), caffeine (Wako Chemicals), theophylline (Kanto Chemicals) and forskolin (Sigma Chemical).

All the experiments were carried out at room temperature (22–26°C).

Results

Changes of the state of the erythrophores in a physiological saline solution

Four types of chromatophores are observed in the dermis of the scale preparation; melanophores, erythrophores, xanthophores and iridophores which are non-motile. The melanophores assumed a state of pigment dispersion in the physiological saline solution. The erythrophores, however, failed to keep a fully dispersed state of the pigment in the saline solution: Beginning to aggregate irregularly, they changed to a

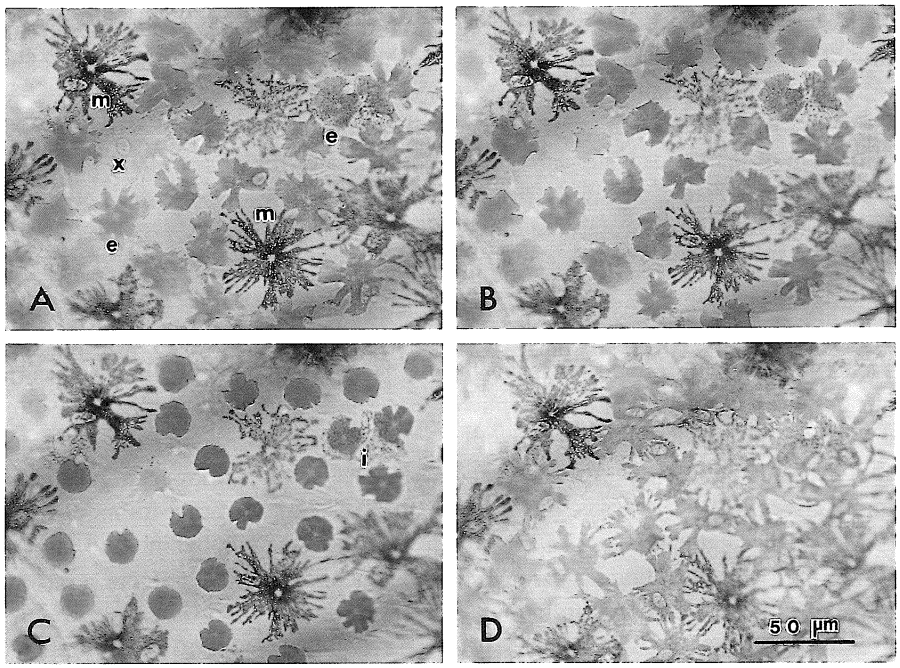

Fig. 1. Changes of the state of erythrophores in an isolated scale from *Halichoeres tenuispinnis* in immersion in a physiological saline solution. A-C, 15, 30 and 90 min after isolation of the scale. D, 10 min after treatment with 10^{-7} M isoproterenol. The treatment was done at 120 min after isolation of the scale. e, erythrophore. i, iridophore. m, melanophore. x, xanthophore.

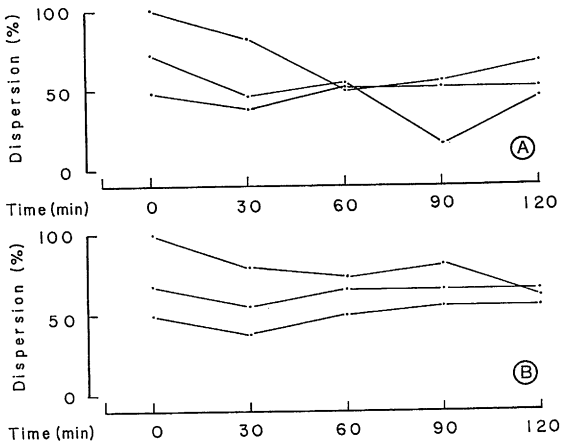

Fig. 2. Change of the state of erythrophores in isolated scales from *H. tenuispinnis* in immersion in a physiological saline solution under a light condition (2,500 lux) (A) and under a dark condition (B).

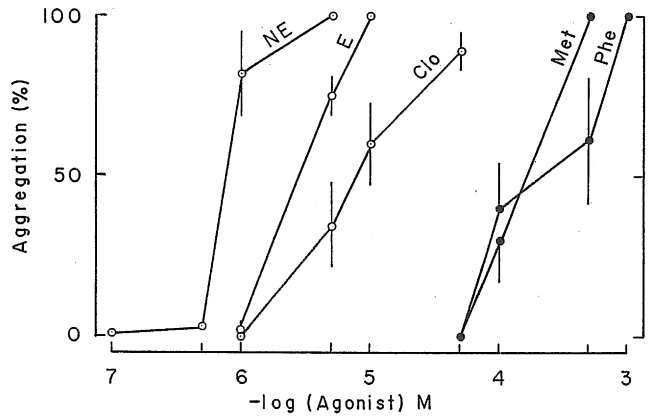

Fig. 5. Concentration-response relation for the pigment aggregating effect of alpha adrenergic agonists on erythrophores in isolated scales from *H. tenuispinnis*. Agonists were applied for 10 min. Each point is the mean of 10 measurements on three different scales. Vertical lines indicate SDs. NE, norepinephrine. E, epinephrine. Clo, clonidine. Met, methoxamine. Phe, phenylephrine.

$ED_{s_{50}}$ estaimed from the curves were as follows: NE(7.6×10^{-7} M)>E(2.9×10^{-6} M)>Clo(6.5×10^{-6} M)≫Met(1.6×10^{-4} M)≧Phe(2.2×10^{-4} M). All the agonists showed a full pigment aggregation action, indicating that they were all full agonists.

Effects of adrenergic antagonists on norepinephrine-induced pigment aggregation

Effects of yohimbine ($alpha_2$ adrenergic antagonist) and corynanthine ($alpha_1$ adrenergic antagonist) on norepinephrine-induced pigment aggregation of erythrophores were examined. In this experiment, 5×10^{-6} M norepinephrine was used, whose concentration was enough to induce a full aggregation of erythrophores (cf. Fig. 5). Following pretreatment with isoproterenol, the erythrophores were treated with single concentrations of each antagonist for 5 min and then were incubated for 10 min in norepinephrine solution containing the antagonist.

Figure 6 shows the relation between concentrations of antagonists and their inhibitory effects on pigment aggregation response of erythrophores to norepinephrine. The ED_{50} of each antagonist for inhibiting norepinephrine-induced aggregation was estimated to be 2.6×10^{-7} M in yohimbine and 7.2×10^{-5} M in corynanthine, respectively, indicating that inhibitory effect of yohimbine was about 280 times higher than that of corynanthine.

Effects of melatonin

In examining a dispersing response of cells, it is necessary to keep the cells in an aggregated state. For this purpose, pigment aggregating effects of melatonin on erythrophores were examined. Melatonin induced a pigment aggregation of the

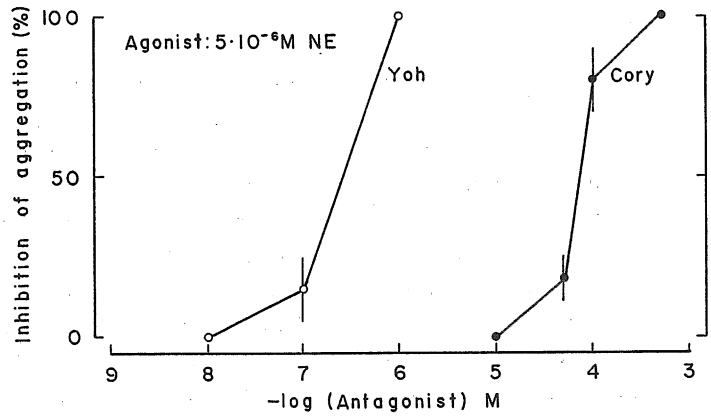

Fig. 6. Inhibition of norepinephrine (5×10^{-6} M)-induced pigment aggregation of the erythrophores by alpha adrenergic antagonists, yohimbine (Yoh) and corynanthine (Cory). Scales were pretreated with either antagonist at various concentrations for 5 min. Then, 5×10^{-6} M norepinephrine was applied for 10 min in the presence of the antagonist. Each point is the mean of 10 measurements on three different scales and vertical lines indicate SDs.

erythrophores. The effect was dose-dependent and a full aggregation was obtained in the concentration of 10^{-6} M. Figure 7 shows the concentration-response relation for a pigment aggregating response of the erythrophores to melatonin.

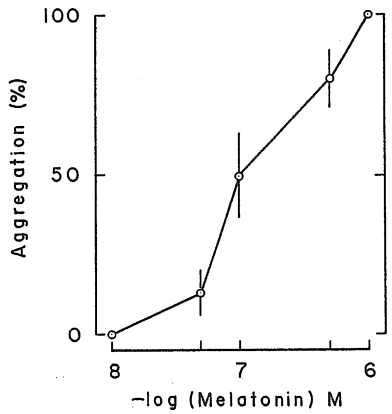

Fig. 7. Concentration-response relation for the pigment aggregating effect of melatonin on erythrophores in isolated scales from *H. tenuispinnis*. Each point is the mean of 10 measurements on three different scales and vertical lines indicate SDs.

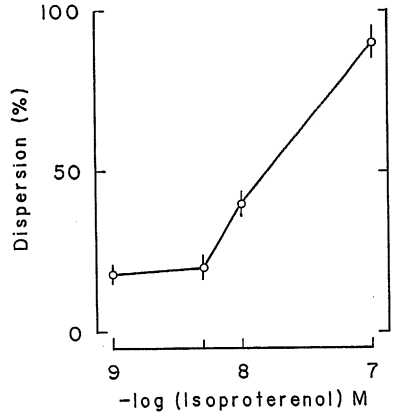

Fig. 8. Concentration-response relation for the pigment dispersing effect of isoproterenol on erythrophores in isolated scales from *H. tenuispinnis*. Isoproterenol was applied for 10 min in the presence of 10^{-6} M melatonin.
Each point is the mean of 10 measurements in three different scales and vertical lines indicate SDs.

Effects of isoproterenol

Melatonin (10^{-6} M) induced a full aggregation of the pigment in the erythrophores within 5 min. The erythrophores, however, began to disperse their pigment soon after the return to the physiological solution. So, the experiment on dispersing effects of isoproterenol were done in the presence of melatonin.

Various concentrations of isoproterenol were applied for 10 min to the erythrophroes in an aggregated state, which had been induced by pretreatment with 10^{-6} M melatonin for 5 min, in the presence of melatonin and the degree of dispersion was calculated. The concentration-response relation for the pigment dispersing effect of isoproterenol is illustrated in Fig. 8. As shwon in this figure, the effect was dose-dependent and an almost full dispersion was obtained at 10^{-7} M.

Effects of adrenergic antagonists on isoproterenol-induced pigment dispersion

Yohimbine, as an alpha antagonist, and propranolol, as a beta antagonist, were used in this experiment. Erythrophores were pretreated with either antagonist in the presence of melatonin for 5 min. The aggregating effect of melatonin was not affected

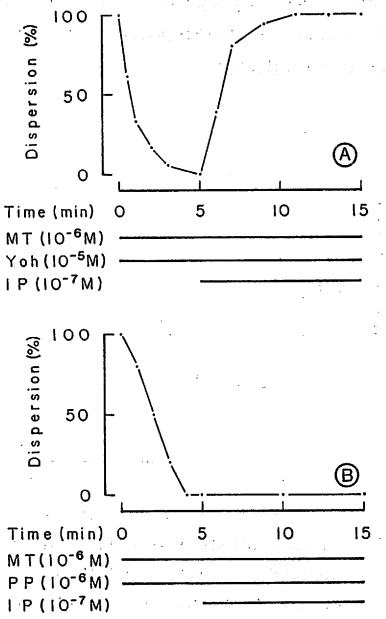

Fig. 9. Effects of yohimbine (Yoh), an alpha adrenergic antagonist (A), and propranolol (PP), a beta adrenergic antagonist (B), on isoproterenol (IP)-induced pigment dispersion of the erythrophores. MT, melatonin.

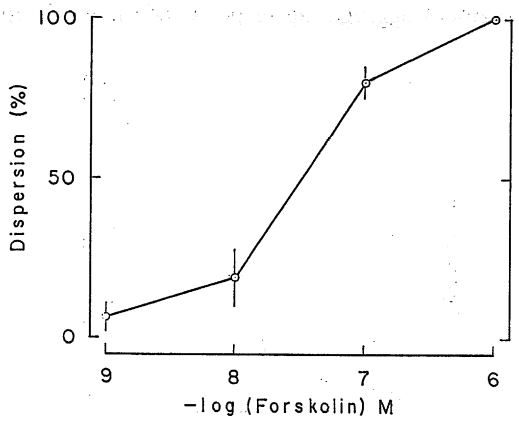

Fig. 10. Concentration-response relation for the pigment dispersing effect of forskolin on erythrophores in isolated scales from *H. tenuispinnis*. After treatment with 10^{-6} M melatonin for 5 min, forskolin at various concentrations was applied for 10 min in the presence of melatonin. Each point is the mean of 10 measurements on three different scales and vartical lines indicate SDs.

by yohimbine nor propranolol, the erythrophores attained to a full aggregation within 5 min. Then, isoproterenol (10^{-7} M) was applied in the presence of melatonin and either antagonist. Yohimbine could not inhibit the dispersing effect of isoproterenol (Fig. 9A), whereas propranolol inhibited effectively the dispersing effect of the drug (Fig. 9B).

Effects of forskolin

In order to assess a posible involvement of an adenylate cyclase system and cAMP, as a second messenger, in the erythrophroes, the effects of forskolin were examined. In this experiment also, forskolin was applied in the presence of 10^{-6} M melatonin to erythrophroes, which had been aggregated by pretreatment with melatonin.

Forskolin induced a dispersion of the pigment in the erythrophroes in a dose-dependent manner. Figure 10 shows the concentration-response relation for the dispersion of the pigment by forskolin. The concentration for inducing a discernible dispersion and that for inducing the full dispersion of the pigment was about 10^{-9} M and 10^{-6} M, respectively.

Discussion

It is well known that, in teleost fish, melanophores in excised preparations assumed a fully dispersed state of the pigment in a physiological saline solution for a few hours (Iga, 1975). The state was designated as the initial dispersion phase by Iwata *et al.* (1959). Generally, physiological experiments on melanophores are designed within this phase. Erythrophorers in isolated scales of a squirrel fish *Myripristis occidentalis* and of a cuckoo wrasse *Labrus ossifagus* maintain a fully dispersed state in the saline solution (Karlsson *et al.*, 1988; Mårtensson *et al.*, 1990). The same statement was true for xanthophores in isolated scales of *Oryzias latipes* (Iga, 1969). The situation was peculiar in *Holocentrus* erythrophores; the erythrophores in a scale pulled from the fish continued to pulsate (*i.e.* aggregate and disperse their pigment granules cyclically) for hours (Byers and Porter, 1977; Porter and McNiven, 1982; Ip *et al.*, 1984).

In the present material *Halichoeres tenuispinnis,* the erythrophoes in isolated scales began to aggregated irregularly and most of them assumed a state of half aggregation of the pigment. Such a pigmentary state was induced similarly under the light and the dark. We know that erythrophorers in some other fish species asumme such a state. In experiments using such materials, it is necessary to keep a state of the pigment stable. The present experiments showed that isoproterenol (10^{-7} M) dispersed the pigment of *Halichoeres* erythrophores without serious inhibitory effects and that its dispersing effect continued for at least 30 min after cessation of the stimulation. Thus, this method may be useful for physiological researches on erythrophores which can not keep their dispersed state in the physiological saline solution after removal from animals.

When a light stimulation was applied to *Halichoeres* erythrophores which had been

kept in the dark, the erythrophorers responded to the stimulation with a rapid aggregation of the pigment. The aggregation was temporary and the erythrophores recovered to the initial state within at least 3 min. The light response was not observed in erythrophores pretreated with 10^{-7} M isoproterenol. Wakamatsu (1978) reported that cultured melanophores from embryos and young fishes of the black platyfish, *Xiphophorus maculatus,* responded to light with melanosome aggregation.

It is recognized that in many teleost fishes adrenoceptors mediating melanosome aggregation within melanophores are alpha in nature (cf. Fujii and Oshima, 1986). Recently, subclassification of alpha adrenoceptors have been made on chromatophores of fishes: The adrenoceptors have been shown to be an $alpha_2$ subtype in melanophores of *Labrus ossifagus* (Andersson *et al.,* 1984), *Oryzias latipes* (Morishita, 1987) and *Labrus berggylta* (Svensson *et al.,* 1989).

Luby-Phelps and Porter (1982) have suggested that aggregating effects of epinephrine on erythrophores of *Holocentrus ascensionis* are mediated by alpha-adrenoceptors, like those of melanophores. In the squirrel fish (*Myripritis occidentalis*), recently, Karlsson *et al.* (1988) presented pharmacological evidnece that alpha adrenoceptors mediating pigment aggregation of the erythrophores are $alpha_2$ in nature. Mårtensson *et al.* (1990) compared the regulation of pigment migration in erythrophores and melanophores of *Labrus ossifagus.* They reported that both types of chromatophores were regulated by $alpha_2$ adrenoceptors and that there were differences in the $alpha_2$ adrenoceptor systems regulating pigment migration in erythrophores and melanophores.

In the present paper, we present pharmacological evidence that pigment aggregation in *Halichoeres* erythrophores is controlled by $alpha_2$ adrenoceptors. Since pigment aggregating potencey of clonidine, known as a specific $alpha_2$ agonist, was far greater than that of specific $alpha_1$ agonists, *i.e.,* methoxamine and phenylephrine. Furthermore, the inhibitory effect of yohimbine, a specific $alpha_2$ antagonist, on the erythrophore response to norepinephrine was about 280 times greater than that of corynanthine, specific $alpha_1$ antagonist.

It has been recognized that beta adrenoceptors mediate pigment dispersion within melanophores in teleost fish (Reed and Finnin, 1972; Miyashita and Fujii, 1975; Finnin *et al.,* 1976; Iga, 1983). Recently, a characterization of the beta adrenoceptors has been made on chromatophores of fishes. Morishita *et al.* (1985) reported that, in *Oryzias latpes,* beta adrenoceptors of melanophores were classified into $beta_2$ subtype and those of leucophores are classifed into $beta_1$ subtype. From a detailed pharmacological analysis, Katayama *et al.* (1990) have claimed that melanophores of the goby, *Tridentiger obscurus* possess both subtypes of beta adrenoceptors, $beta_1$ and $beta_2$.

Little information, however, is available as to beta adrenoceptors in fish erythrophores. Matsumoto *et al.* (1978) showed that erythrophores of the swordtail possess beta adrenoceptors mediating dispersion of pigmentary organelles. In the present experiments, the dispersion response evoked by isoproterenol was inhibited by

propranolol, a beta antagonist, but not by yohimbine, an alpha antagonist, indicating that adrenoceptors mediating pigment dispersion are of a beta type.

Forskolin, an activator of the catalytic subunit of adenylate cyclase, increases intracellular levels of cAMP in a variety of eukaryotic cells (Seamon and Daly, 1981; Sano *et al.*, 1983). So, it has served as an invaluable tool for investigators into the role of cAMP in physiological responses to hormones and transmitters. Forskolin acted effectively to disperse the pigment within the erythrophorers of *Halichoeres tenuispinnis,* suggesting that increases in levels of intracellular cAMP elicit the dispersion of the pigment in the erythrophores, as in other types of chromatophores in poikilothermal vertebrates.

In summary, the present results demonstrate that the erythrophores of *Halichoeres tenuispinnis* possess alpha adrenoceptors of an $alpha_2$ subtype which mediate pigment aggegation and beta adrenoceptors mediating pigment dispersion. The stimulation of the beta receptors activates adenylate cyclase, which, in turn, causes an elevation of intracellular levels of cAMP.

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