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Alpha Adrenoceptors: Pigment Aggregation in *Oryzias* Leucophores

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ABSTRACT

An *in vitro* method for assessing pigment aggregation in leucophores is described, in which isolated scales of the medaka, *Oryzias latipes*, were utilized. Theophylline produced reversible pigment dispersion in the leucophores. The pigment dispersion could not be inhibited by alpha and/or beta adrenergic blocking agents. Catecholamines, noradrenaline, adrenaline and isoproterenol, induced pigment dispersion in the leucophores which assumed a moderate pigment dispersion under the influence of theophylline. Dibenamine could not modify the pigment-dispersing effect of the catecholamines in the presence of theophylline. Whereas propranolol reversed the response of leucophores to the catecholamines; the leucophores responded with a rapid pigment aggregation in response to the amines. Agonistic ranking experiments showed that the relative effectiveness of the amines in causing pigment aggregation was: adrenaline>noradrenaline>isoproterenol. These catecholamine-induced pigment aggregation responses were inhibited by dibenamine. These results indicate that the leucophores of *Oryzias latipes* possess alpha adrenoceptors, which mediate the pigment aggregation.

Introduction

Besides melanophores and xanthophores, leucophores are found in the dermis of the medaka, *Oryzias latipes* (wild type). The leucophores contain an abundance of light-reflecting granules, leucosomes, which can move centrifugally or centripetally within the cells. These cells have been observed in rather few species of fishes, *e.g.*, *Fundulus heteroclitus* (Odione, 1933; Fries, 1942; Menter *et al.*, 1979), *F. majalis* (Fries, 1942), *Cyprinodon variegatus* (Fries, 1942) and *Lebistes reticulatus* (Takeuchi, 1979).

It has been shown that, either *in vitro* or *in vivo*, the direction of the motile response of leucophores is opposite to that of the motile response of other chromatophores, melanophores and xanthophores: When the leucosomes disperse within the cells, the pigment granules in the others aggregate in the centers of the cells, and *vice versa* (Fries, 1942; Miyoshi, 1952; Kinoshita, 1953; Obika, 1976; Iga *et al.*, 1977; Iga, 1978; Fujii and Miyashita, 1979). Because of the opposite motile response, investigations on the pigment migratory mechanism and its regulatory system of leucophores have

become of special interest lately.

In previous investigations, the leucophores of *Oryzias latipes* have been shown to be under adrenergic control, and to possess beta adrenoceptors which mediate pigment dispersion (Iga *et al.*, 1977; Iga, 1977). On the other hand, no information is known about any regulatory mechanisms of pigment aggregation up to the present day.

The purpose of this paper is to describe a possible participation of adrenoceptors in the rapid aggregation of leucosomes within the cells, and to characterize the nature of the receptors concerned.

Materials and Methods

The wild type of the medaka, *Oryzias latipes*, was used as the experimental material. A scale isolated from the trunk of the fish was held, epidermal side down, on the under surface of a cover glass, which was set on a glass chamber which was filled with a physiological saline solution of the following composition: 128 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM Tris-HCl buffer (pH 7.2). A KCl solution was isotonic with the physiological saline (pH=7.2 by 5.0 mM Tris-HCl buffer). The KCl solution induced dispersion of pigment granules in the leucophores (Miyoshi, 1952; Iga, 1978).

The following drugs were used: *l*-noradrenaline hydrochloride (Sigma Chemical, St. Louis), *l*-adrenaline hydrochloride (Sankyo, Tokyo), *dl*-isoproterenol hydrochloride (Sigma Chemical, St. Louis), dibenamine hydrochloride (Tokyo Kasei, Tokyo), propranolol hydrochloride (Sigma Chemical, St. Louis) and theophylline (Tokyo Kasei, Tokyo). All drugs were dissolved in the physiological solution. As a solution of adrenaline, injection fluid was used for convenience, which was diluted with the saline immediately before use.

It is rather difficult to distinguish leucophores from melanophores when an ordinary transmission light microscope is employed for observation, since leucophores are commonly found to exist closely associated with melanophores and, moreover, they are brown in appearance in transmitted light. Therefore, a dark-field epi-illumination microscope (Olympus, Neopak BHA–312 NE) was employed for observation of the leucophores. By using this type of microscope, leucophores were clearly visible on the dark background. Moreover, the combined use of a transmission illumination with epi-illumination enabled us to observe leucophores and other chromatophores simultaneously. The use of epi-illumination was limited to the period when the measurement was made, because it was found that the continuous illumination induced a pigment dispersion in some of the leucophores, as previously pointed out (Iga *et al.*, 1977; Iga, 1978).

The response of the leucophores was expressed as change in the length of a given cellular branch of a leucophore. The measurement of the length of a branch was made by means of an ocular micrometer.

Experiments were done at room temperature (23–26°C).

Results

Detection of pigment-aggregating effect

To examine whether a certain substance has a pigment-aggregating effect or not, it is necessary to keep the cells at a moderate pigment-dispersed state, since leucophores in isolated scales of fish remain in a state of pigment aggregation in the physiological saline. At the present experiments, theophylline, an inhibitor of a specific phosphodiesterase that converts cyclic AMP to noncyclic AMP, was conveniently employed as a pigment-dispersing agent, because the pigment dispersion induced by theophylline was inhibited by neither beta adrenergic blocking agents, nor alpha adrenergic blocking agents, as will be shown in the following experiments (Fig. 3 and 4).

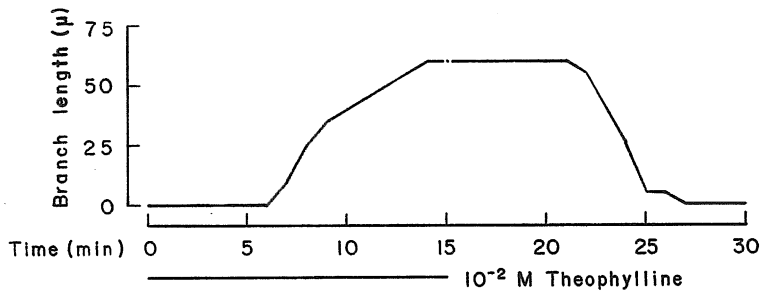

Fig. 1. Pigment-dispersing effect of theophylline on *Oryzias* leucophores.

In strong solution (*e.g.*, 10^{-2} M) of theophylline, the leucosomes became dispersed to a high degree, which was maintained during the theophylline treatment (Fig. 1). When the solution of theophylline was replaced with the physiological saline, the leucophores became aggregated within 10 to 15 minutes after a quiescent period of few minutes: Recovery from the theophylline effect was rather rapid.

Under its coexistence, therefore, the possible pigment-aggregating effect of catecholamines was examined. In most experiments, a 10^{-2} M solution of theophylline was used as a solution for inducing leucosome dispersion.

Effect of catecholamines

When various concentrations (10^{-6}–10^{-5} M) of noradrenaline solution were applied to the leucophores which maintained pigment dispersion under the effect of theophylline, the leucophores kept their dispersed state, or they caused a further dispersion of pigment (Fig. 2). The similar results were obtained in the cases of other catecholamines, adrenaline and isoproterenol.

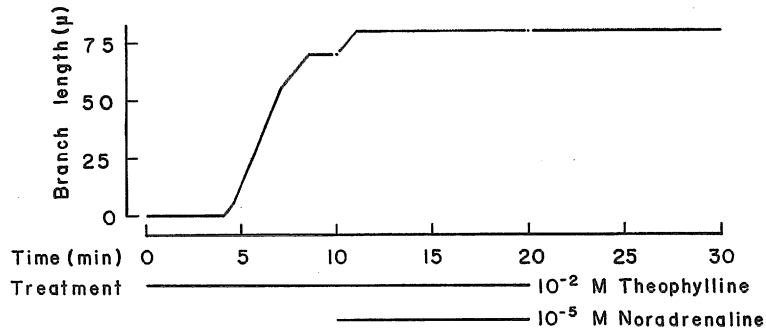

Fig. 2. Response of *Oryzias* leucophores to noradrenaline in the presence of theophylline.

Effect of an alpha adrenergic blocker

Leucophores were incubated in a solution of 10^{-4} M dibenamine, an alpha adrenergic blocker, for 10 minutes prior to the treatment with theophylline. The pigment dispersion in the leucophores induced by theophylline could not be affected by the pretreatment with dibenamine. As is shown in Fig. 3, when noradrenaline was applied to the leucophores, in which leucosomes had been moderately dispersed under the influence of 10^{-2} M theophylline, a further dispersion of leucosomes was observed. Fundamentally identical results were obtained when the other catecholamines, adrenaline and isoproterenol, were applied to the leucophores under the influence of theophylline.

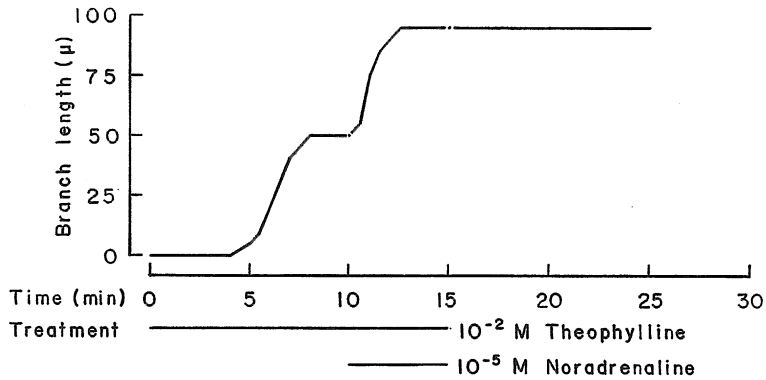

Fig. 3. Effect of dibenamine on the response of *Oryzias* leucophores to noradrenaline. The leucophores were incubated in a solution of 10^{-4} M dibenamine for 10 min prior to theophylline application. The treatment with dibenamine is not drawn in this figure.

Effect of a beta adrenergic blocker

Following a 15-min treatment with 10^{-4} M propranolol, a beta adrenergic blocker,

the leucophores were treated with 10^{-2} M theophylline. Theophylline brought about normally the pigment dispersion in the leucophores. The leucophores, however, responded with a rapid pigment aggregation in response to noradrenaline after the treatment with propranolol. A typical example of the effect of 10^{-5} M noradrenaline is shown in Fig. 4; here, noradrenaline could induce a complete pigment aggregation within 3 minutes. Adrenaline and isoproterenol also produced the pigment aggregation in the leucophores pretreated with propranolol. However, the pigment-aggregating action of isoproterenol was weaker than that of the other catecholamines. Quantitative analysis of the pigment-aggregating effect of three catecholamines will be shown in the next experiment.

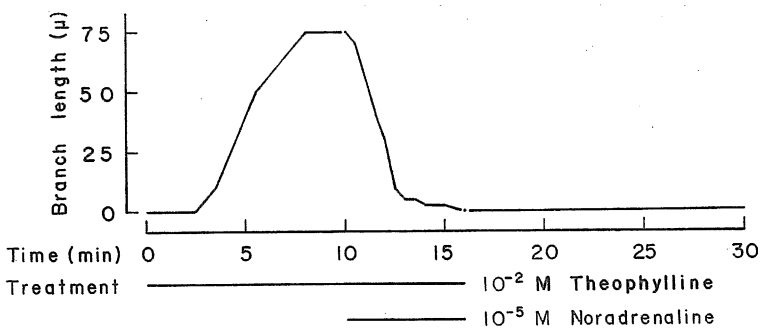

Fig. 4. Effect of propranolol on the response of *Oryzias* leucophores to noradrenaline. The leucophores were incubated in a solution of 10^{-4} M propranolol for 15 min prior to theophylline application. In this figure the treatment with propranolol is not drawn.

Pigment-aggregating effect of catecholamines

An experimental procedure for examining the pigment-aggregating activity of the catecholamines was identical with that described in the previous section. The degree of pigment aggregation was taken as the difference between the length of a given branch of a leucophore before and after a 10-min treatment with each catecholamine. Results were expressed as a 'relative pigment-aggregating effect' by expressing the drop (shown as distance 'C' in Fig. 5) as a percentage of the branch length just before application of a catecholamine (distance 'B' in Fig. 5), *i.e.*, relative pigment-aggregating effect $= C/B \times 100$. The pigment-aggregating effect at various concentrations of three catecholamines was examined. The results are illustrated in Fig. 6 as concentration-response relations, where the percent response of leucophores is plotted to the molar concentration of catecholamines.

As indicated in the figure, the minimal concentration required for producing discernible pigment aggregation in the leucophores was found to be about 5×10^{-8} M for adrenaline, 5×10^{-7} M for noradrenaline and 5×10^{-6} M for isoproterenol, respec-

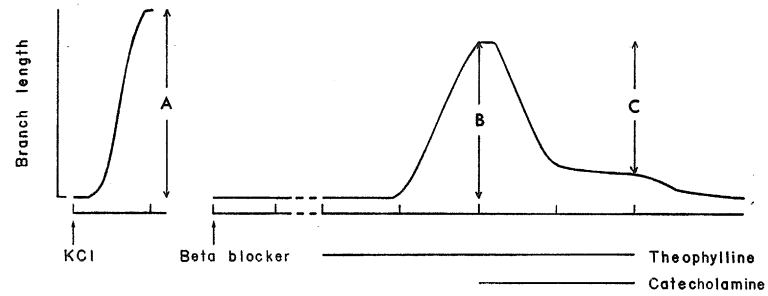

Fig. 5. Assessment of pigment-aggregating effect of catecholamines on *Oryzias* leucophores. The pigment-aggregating effect of the agents was expressed as $C/B \times 100$, where 'B' is the length of a given branch of a test leucophore just before application of catecholamines and 'C' is the difference between 'B' and the branch length of the leucophore at 10 min after application of catecholamines. 'A' is the full extended branch length of the leucophore in the KCl solution. Pigment-dispersing effect of theophylline is indicated as $B/A \times 100$.

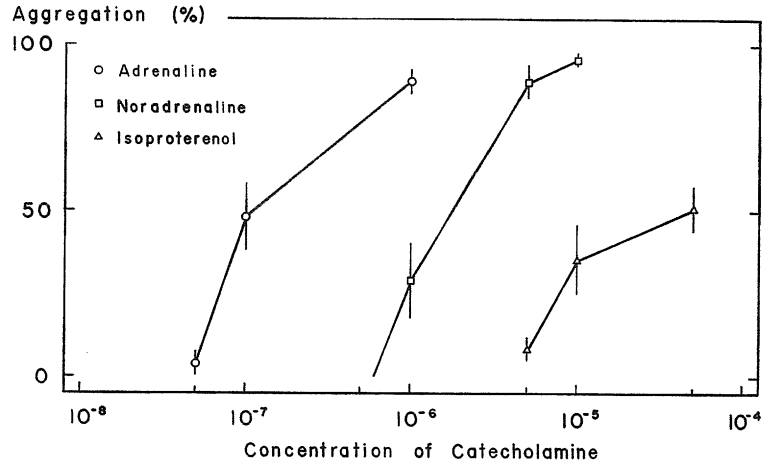

Fig. 6. Concentration-response relations of three catecholamines on the pigment-aggregating response of *Oryzias* leucophores. The amines were applied to the leucophores in the presence of 10^{-2} M theophylline. Vertical bars indicate standard deviations.

tively. Full dispersion of pigment was obtained at 10^{-6} M for adrenaline and at 10^{-5} M for noradrenaline. Pigment aggregation by isoproternol could not exceed a level of about 50% even at a high concentration (5×10^{-5} M). Thus, the order of relative effectiveness of catecholamines for causing pigment aggregation was adrenaline > noradrenaline > isoproterenol.

Inhibition of pigment-aggregating effect of catecholamines

In order to demonstrate that the catecholamine-induced pigment aggregation is actually produced through their interaction with alpha adrenoceptors, the influence of a specific antagonist was examined. As an alpha adrenergic blocker, dibenamine was used in this experiment.

Following treatment with 10^{-4} M propranolol for 15 minutes, 10^{-4} M dibenamine was applied to the leucophores for 10 minutes. After a series of these treatments, the leucophores were exposed to 10^{-2} M theophylline solution for 10 minutes in order to bring about a state of pigment dispersion. The solution was then changed to a theophylline solution containing 10^{-6} M adrenaline to examine the aggregating effect of the agent. The aggregating action of adrenaline was effectively inhibited by the pretreatment with dibenamine, as is shown in Fig. 7. A slow dispersion of pigment in the leucophores was observed in the test solution. This pigment dispersion may depend on the effect of theophylline.

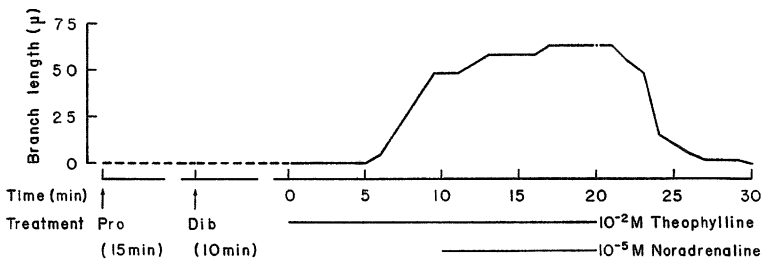

Fig. 7. Effect of dibenamine (Dib. 10^{-4} M) on the pigment-aggregating effect of noradrenaline. Pro, 10^{-4} M propranolol.

When the test solution was changed to the physiological saline, the leucophores returned to the initial aggregated state within 10 minutes. The time of recovery was identical with that from the treatment with theophylline alone. It should be mentioned that the pretreatment with dibenamine also completely inhibited the aggregating effect of noradrenaline.

Discussion

It has been observed that theophylline produces pigment dispersion in some chromatophore systems, *e.g.*, fish melanophores (Abramowitz and Chavin, 1974), amphibian melanophores (Abe *et al.*, 1969; Goldman and Hadley, 1970) and fish leucophores (Obika, 1976; Yamada and Iwakiri, 1977).

In the present experiments, theophylline produced leucosome dispersion which was reversible. The theophylline-induced pigment dispersion could not be inhibited

by alpha and/or beta adrenergic blocking agents, indicating that theophylline does not act through stimulation of adrenergic receptors. The recent concept shows that theophylline produces the pigment dispersion in melanophores by inhibiting a specific phosphodiesterase that degrades cyclic AMP, thereby allowing an increase in endogenous cyclic AMP levels within chromatophores (see Bagnara and Hadley, 1973). Recently, Yamada and Iwakiri (1977) observed that cyclic AMP induced a pigment dispersion in leucophores of *Oryzias latipes*. From these reasons theophylline was conveniently employed as a pigment dispersing agent for studying adrenergic receptor mechanisms of fish leucophores.

Catecholamines induced the pigment dispersion in fish leucophores through the stimulation of beta adrenoceptors (Iga *et al.*, 1977). The effect of catecholamines could not be modified in the presence of theophylline, nor by treatment of leucophores with an alpha adrenergic blocker, whereas after treatment with a beta adrenergic blocker, these catecholamines produced a rapid pigment aggregation in the leucophores. It is rather important to mention that the catecholamine-induced pigment aggregation was effectively antagonized by pretreatment with an alpha adrenergic blocker. These results indicate that alpha adrenoceptors are present in *Oryzias* leucophores in addition to beta adrenoceptors mediating pigment dispersion, and that they take part in the pigment aggregation in the leucophores. The order of the effectiveness of catecholamines in causing pigment aggregation was: adrenaline > noradrenaline > isoproterenol, a characterization of an alpha adrenergic receptor mediated response, being the same with that in melanosome-aggregating activity of these amines in melanophores of the same species (Iga, 1968).

Catecholamines stimulate both alpha and beta adrenoceptors. Since in leucophores beta adrenoceptors predominate over alpha adrenoceptors mediating pigment aggregation, it would appear that the effect of beta adrenoceptor stimulation dominates and this results in pigment dispersion. While in the presence of blockade of beta adrenoceptors it is possible that there would be sufficient alpha activity left to cause pigment aggregation.

Although *in vivo* regulatory mechanisms between these antagonistic adrenoceptors still remain to be clarified, it should be emphasized that alpha stimulants, especially adrenaline, effectively produced pigment aggregation at low concentrations that could not produce any pigment dispersion in the leucophores.

Acknowledgment

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Literature

Abe, K., R. W. Butcher, W. E. Nicholson, C. E. Baird, R. A. Liddle and G. W. Liddle, 1969. Adenosine 3′, 5′-monophosphate (cyclic AMP) as the mediator of the actions of melanocyte stimulating hormone (MSH) and norepinephrine on the frog skin. *Endocrinology,* **84**, 362–368.

Abramowitz, J. and W. Chavin, 1974. In vitro response of goldfish (*Carassius auratus* L.) dermal melanophores to cyclic 3′, 5′-nucleotides, nucleoside 5′-phosphates and methylxanthines. *J. Cell Physiol.,* **84**, 301–310.

Bagnara, J. T. and M. E. Hadley, 1973. Chromatophores and color change. 132 p. Prentice Hall, Englewood Cliffs, N. J.

Fries, E. F. B., 1942. White pigmentary effectors (leucophores) in killifishes. *Proc. Nat. Acad. Sci.,* **28**, 396–401.

Fujii, R. and Y. Miyashita, 1979. Photoelectric recording of motile responses of fish leucophores. *Annot. Zool. Japon.,* **52**, 87–94.

Goldman, J. M. and M. E. Hadley, 1969. The beta adrenergic receptor and cyclic 3′, 5′-adenosine monophosphate: Possible roles in the regulation of melanophore responses of the spadefoot toad, *Scaphiopus couchi. Gen. Comp. Endocrinol.,* **13**, 151–163.

————, and ————, 1970. Cyclic AMP and adrenergic receptors in melanophore responses to methylxanthines. *Eur. J. Pharmacol.,* **12**, 365–370.

Hadley, M. E. and J. M. Goldman, 1969. Physiological color changes in reptiles. *Am. Zoologist,* **9**, 489–504.

Iga, T., 1968. Action of catecholamines on the melanophores in the teleost, *Oryzias latipes. Zool. Mag. Tokyo,* **77**, 19–26.

————, 1977. The nervous mechanism controlling pigment dispersion in *Oryzias* leucophores. *Zool. Mag. Tokyo,* **86**, 386 (Abstract, in Japanese).

————, 1978. The mode of action of potassium ions on the leukophores of a freshwater teleost, *Oryzias latipes. J. Exp. Zool.,* **205**, 413–422.

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

Kinosita, H., 1963. Electrophoretic theory of pigment migration within fish melanophore. *Ann. New York Acad. Sci.,* **100**, 992–1004.

Menter, D. G., M. Obika, T. T. Tchen and J. D. Taylor, 1979. Leucophores and Iridophores of *Fundulus heteroclitus*: Biophysical and ultrastructural properties. *J. Morph.,* **160**, 103–120.

Miyoshi, S., 1952. Response of iridocytes in isolated scales of the medaka (*Oryzias latipes*) to chlorides. *Annot. Zool. Japon.,* **25**, 21–29.

Obika, M., 1976. An analysis of the mechanism of pigment migration in fish chromatophores. *Pigment Cell,* **3**, 254–264.

Odiorne, J. M., 1933. The occurrence of guanophores in *Fundulus. Proc. Nat. Acad. Sci.,* **19**, 750–754.

Takeuchi, I. K., 1976. Electron microscopy of two types of reflecting chromatophores (Iridophores and leucophores) in the guppy, *Lebistes reticulatus* Peters. *Cell Tiss. Res.,* **173**, 17–27.

Yamada, K. and M. Iwakiri, 1977. Effects of cyclic AMP, methylxanthines and imidazole on fish leucophores. *Bull. Biol. Sci. Hiroshima Univ.,* **43**, 23–27.