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Subtypes of β-adrenoceptos in the melanophore of *Oryzias latipes* (Teleostei)

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Abstract β-Adrenergic agonists, viz, isoprenaline, noradrenaline, adrenaline, dobutamine, salbutamol and terbutaline each in the presence of melatonin and phenoxybenzamine caused the dispersion of pigment within melanophores in which pigment had been made to aggregate by melatonin in isolated scales of *Oryzias latipes* (a fresh-water teleost). The extent of the dispersion of pigment in the melanophores increased as the external concentration of a β-adrenergic agonist was increased. Assuming that different two types of β-receptors coexist in the melanophores, Hofstee plots for the effects of the β-agonists on the melanophores were analysed with the results that the mean ratio of the percentages of β_1- and β_2-adrenoceptors for the total population of β-receptors was 37.4% : 62.6%, respectively. β-adrenoceptors of the melanophores of *Oryzias latipes* were confirmed to be β_2-type. The possibility for the occurrence of negative cooperative interactions among β-adrenoceptors in the melanophores was discussed.
Key Words: β-adrenergic agonists, pigment-dispersing effects, Hofstee analyses.

Introduction

In many teleost fish, dermal melanophores are controlled by the adrenergic nerves. The melanophores are known to posess both α- and β-adrenoceptors and some other kinds of receptors for various agents in their membranes (Fujii and Oshima, 1986). The aggregation of melanin pigment within the melanophores is evoked by the action of the adrenergic neurotransmitter or exogenous adrenergic agonists on α-adrenoceptors of the melanophores. The aggregation of pigment can be evoked also by the other kinds of agents, for example, melatonin, MCH (melanin concentrating hormone)(Bagnara and Hadley, 1973) and verapamil (Katayama et al., 1990) which act on their respective receptors in the membranes of the melanophores.

By contrast, the dispersion of pigment within the melanophores is brought about by the actions of adrenergic agonists on β-adrenoceptors of the melanophores (Bagnara and Hadley, 1973; Miyashita and Fujii, 1975). The dispersion of pigment is also evoked by some agents other than adrenergic agonists, for example, MSH (melanophore stimulating hormone), adenosine and methylxanthines (Fujii and Oshima, 1986).

Miyashita and Fujii (1975) first revealed that melanophores of a teleost, *Lebistes reticulatus*, the guppy possess β-adrenoceptors in addition to α-adrenoceptors. β-Adrenoceptors in mammalian heart, lung and smooth muscles were classified into two subtypes, β_1- and β_2-receptors (Lands et al., 1967; Minneman and Molinoff, 1980). Lately, in addition to β_1- and β_2-subtypes, the third β-receptors were found in the rat brown-adipocytes (Arch et al., 1984) and in the white-adipocytes in several kinds of mammals (Langen et al., 1991).

With regard to the subtype of β-adrenoceptors in the melanophore of fish, only a little information has been presented so far. Morishita et al. (1985) reported that melanophores of *Oryzias latipes* pos-

sess β_2-adrenoceptors in addition to α_2-adrenoceptors. Katayama et al.(1990) reported that melanophores of a goby, *Tridentiger obscurus* possess both β_1- and β_2-adrenoceptors.

In the present study, we examined the possibility for the existence of the two subtypes of β-adrenoceptors in the melanophore of *Oryzias latipes*.

Materials and Methods

Melanophores in scales isolated from *Oryzias latipes*, the Medaka, were used. The fresh-water fish, *Oryzias latipes* were collected in the rice-fields in Matsue City and reared in the laboratory.

An isolated scale was fastened under a cover glass of a glass chamber placed on the stage of a light-microscope. The isolated scale was first immersed in physiological saline which contained (in mM) NaCl, 128; KCl, 2.6; $CaCl_2$, 1.8; Tris-HCl, 7; $NaHCO_3$, ca 2.4; pH=7.2.

Procedures:

In the present experiments, responses of denervated melanophores to adrenergic agonists were mainly recorded. Denervation of melanophores in an isolated sale was achieved by treating the scale for 10 min with a reserpine solution (10 μM reserpine and 1.25 mM citric acid were added to physiological saline). After the treatment with reserpine, the sacle was repeatedly washed in physiological saline. Melanophores in the reserpinized scale did not aggregate their pigment in response to KCl solution which contained 41 mM KCl, 89.5 mM NaCl and 1.8 mM $CaCl_2$(pH=7.2).

Experiments were made on these denervated melanophores as follows. At first, the aggregation of pigment was evoked within the melanophores by treating the scale with 0.5 μM melatonin in the presence of 5 μM phenoxybenzamine (all these agents were dissolved or diluted with physiological saline). Then the melanophores were treated with a test solution which contained a β-adrenergic agonist, 0.5 μM melatonin and 5 μM phenoxybenzamine. The concentration of the β-agonist, for example, isoprenaline in the test solution, was successively increased step-wise from 1 nM to 500 nM. The melanophores were incubated for 5-10 min in each concentration of the β-adrenergic agonist tested. The responses of two-three melanophores in a given area in the scale were photoelectrically recorded simultaneously according to the method described by Nagahama and Katayama (1985)(Fig. 1).

The extent of a dispersion of pigment within the melanophores was expressed as a percentage of the range between the initial aggregated condition of pigment caused by 0.5 μM melatonin plus 1 μM phenoxybenzamine and the complete dispsersion of pigment. After treatments with a series of test solutions of a β-adrenergic agonist, the scale was washed in physiological saline and the response of the melanophores to 0.1 μM MCH was

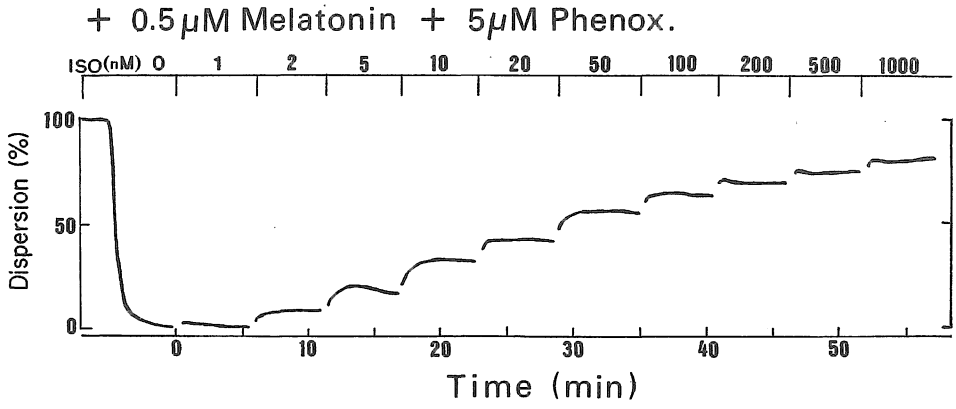

Fig. 1. A photoelectic recording of the responses of melanophores to isoprenaline in the presence of 0.5 μM melatonin and 5 μM phenoxybenzamine in an isolated scale of *Oryzias latipes*. Denervated melanophores were used exclusively. Pigment of the melanophores was initially made to aggregate by melatonin in the presence of phenoxybenzamine. The concentration of isoprenaline was inceased cummulatively as indicated at the top of the figure.

examined. The concentration of MCH at 0.1 μM was previously confirmed to be enough to evoke the full aggregation of pigment in melanophores of *Oryzias latipes*.

Drugs:

Following drugs were used, which were purchased from the companies indicated. (±) Isoprenaline hydrochloride, (−) adrenaline bitartrate, (−) noradrenaline hydrochloride, salbutamol hemisulfate, (±) propranolol hydrochloride, reserpine, melatonin (Sigma Chemical Co., St. Louis, MO, USA); terbutaline sulfate (Bricanyl injection, Fujisawa Pharm. Co. Ltd., Osaka); dobutamine hydrochloride (Dobutrex injection, Shionogi & Co. Ltd., Osaka); phenoxybenzamine hydrochloride (Nakarai Chemical Co. Ltd., Kyoto); MCH (melanin-concentrating hormone, Peninsula Lab. Inc. Belmont, CA, USA; Funakoshi Co. Ltd., Tokyo).

The catecholamines used were dissolved in a 0.5% solution of sodium metabisulfate. Reserpine was dissolved in a 125 mM solution of citric acid. Phenoxybenzamine was dissolved in ethanol. These drug solutions and injections were diluted with physiological saline immediately before use. The other drugs were dissolved in physiological saline shortly before use.

Results

(a) The pigment-dispersing effects of β-adrenergic agonists

The aggregation of melanin pigment was evoked within melanophores by melatonin at 0.5 μM in the presence of 5 μM phenoxybenzamine. The extent of the aggregation of pigment was not full, but about 50-70% of the full aggregation which was determined by the response of the melanophores to 0.1 μM MCH at the final stage of experiments.

(±) Isoprenaline, (−) adrenaline, (−) noradrenaline, salbutamol, terbutaline and dobutamine were all effective in inducing the redispersion of pigment in melanophores in which pigment had been aggregated by melatonin (Fig. 2). The pigment dispersing effects of the adrenergic agonists increased dose-dependently.

The pigment-dispersing effects of these adrenergic agonists on the melanophores were probably attributed to the actions of these agonists on β-adrenoceptors of the melanophores. In order to confirm the actions of the adrenergic agonists on β-adrenoceptors of the melanophores, influence of propranolol which was the β-adrenergic antagonist on the pigment-dispersing effects of isoprenaline and salbutamol was examined. The extent of the pigment-dispersing response of melanophores to 100 nM isoprenaline in the presence of 1 μM propranolol, 0.5 μM melatonin and 5 μM phenoxybenzamine was less than 20% of the full dispersion of pigment. The result similar to the above was obtain-

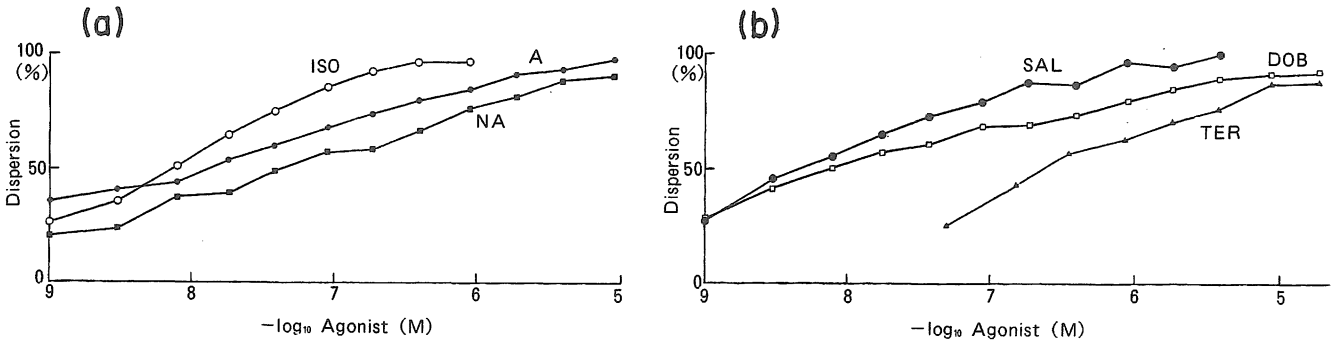

Fig. 2. The pigment-dispersing responses of melanophores to a β-adrenergic agonist in the presence of 0.5 μM melatonin and 5 μM phenoxybenzamine plotted against the concentration of the β-agonist. (a) β-agonist was isoprenaline (ISO), adrenaline (A) or noradrenaline (NA). (b) β-agonist was salbutamol (SAL), dobutamine (DOB) or terbutaline (TER). Each point represents the mean of 8-10 measurements.

ed with regard to the effect of 100nM salbutamol. Thus, the effects of these β-adrenergic agonists were considerably inhibited by propranolol.

These results indicated that the pigment-dispersing effects of isoprenaline and salbutamol were due the actions of these agonists on β-adrenergic receptors of the melanophores. The effects of the other β-adrenergic agonists used on the

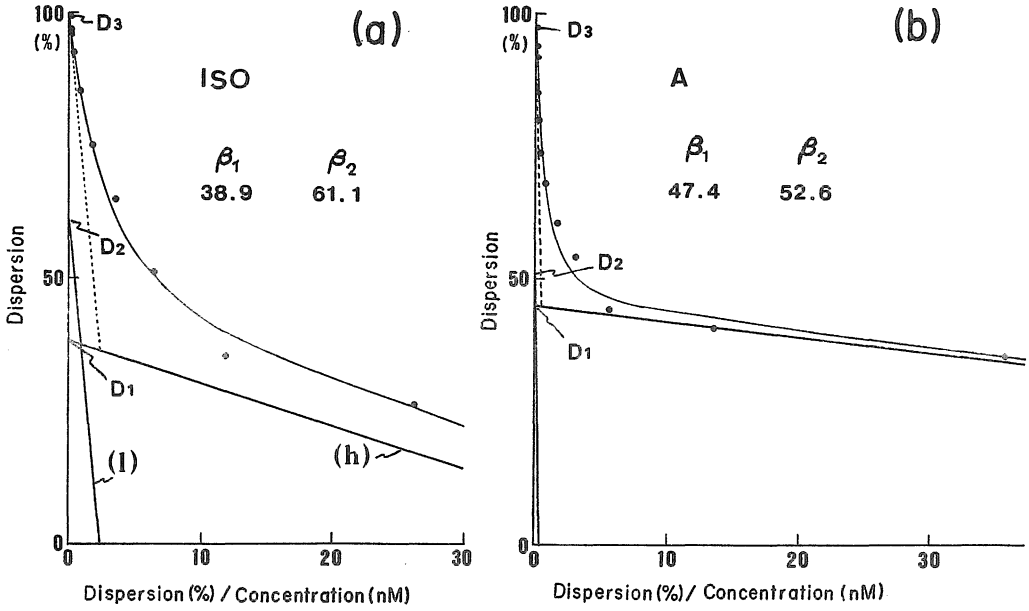

Fig. 3. Hofstee plots for the pigment-dispersing effects of isoprenaline (a) and adrenaline (b) on melanophores. Data were transformed from the concentration-response curves presented in Fig. 2. Ordinate: The extent of the pigment-dispersion in %. Abcissa: the extent of the pigment-dispersion ÷ the concentration of a β-agonist. D_1: y-axis-intercept of the asymptote of a high affinity-component (h). D_2: y-axis-intercept of the asymptote of a low affinity-component (l). D_3: y-axis-intercept of the dotted line indicating $D_1 + D_2 = D_3$. β_1, β_2 with numbers indicate the percentages of β_1- and β_2-receptors to the total population of β-receptors.

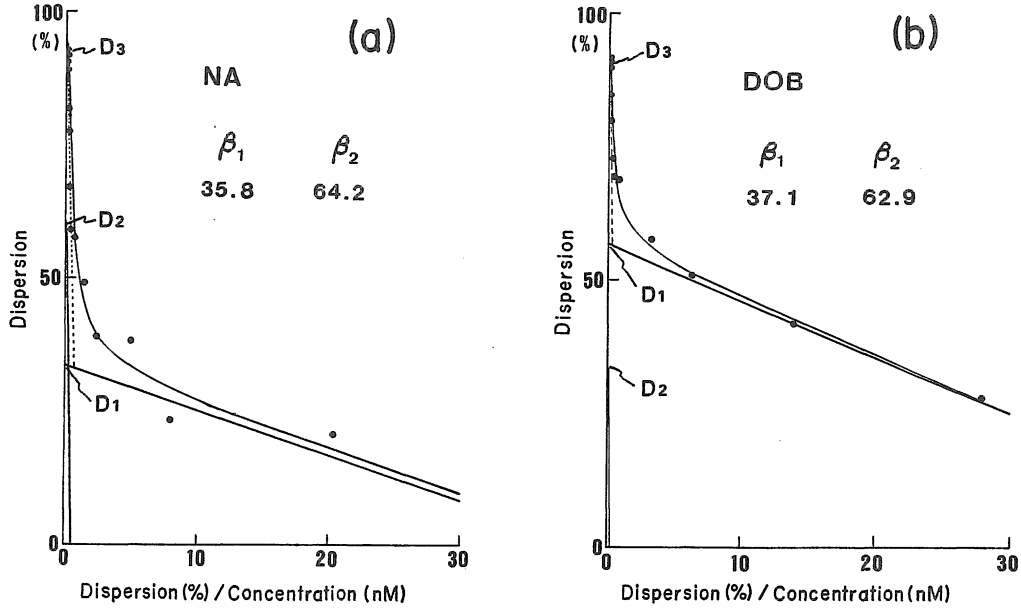

Fig. 4. Hofstee plots for the pigment-dispersing effects of noradrenaline (a) and dobutamine (b) on melanophores. Explanations for β_1, β_2, D_1, D_2 and D_3 in this and the following figures are the same to those described in Fig. 3.

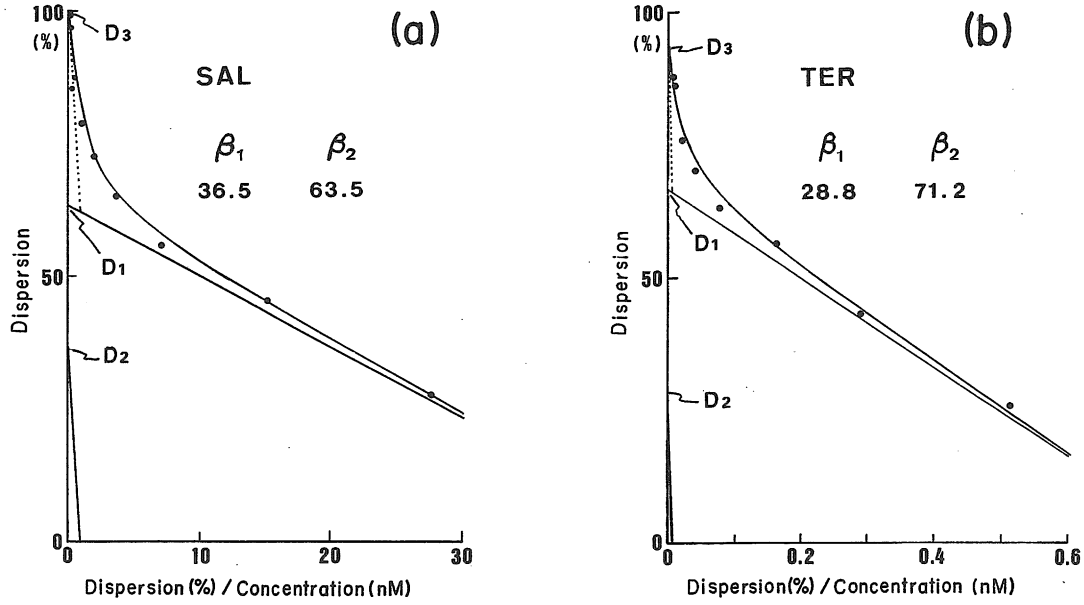

Fig. 5. Hofstee plots for the pigment-dispersing effects of salbutamol (a) and terbutaline (b) on melanophores.

melanophores can be considered to be due to the actions of the β-agonists on β-adrenoceptors of the melanophores.

(b) Hofstee analyses of the effects of β-agonists

Hofstee plots (or modified Scatchard plots) were made from the data presented in the concentration-response curves for the effects of the β-agonists on the melanophores. All of the Hofstee plots for the effects of the β-agonists were curvilinear with upward concavity (Figs. 3, 4 and 5).

It is known that a concave-upward curve of Hofstee plot for the effect of an adrenergic agonist or antagonist on adrenoceptors suggests either the existence of heterogenous binding sites with different affinities or the existence of negative cooperative interactions among the binding sites, or both (Limbird and Lefcowitz, 1979; Mendel et al., 1985).

There is possibility that negative cooperativity exists among β-adrenoceptors of melanophores. On the other hand, it is also possible that heterogeneous receptors with discrete affinities for a β-adrenergic agonist exist in the membrane of the melanophore, since β_1-selective agonist noradrenaline and β_2-selective agonists salbutamol and terbutaline are all effective in evoking the dispersion of pigment in the melanophores. Moreover, it has been suggested that the melanophores of the goby, *Tridentiger obscurus* possess both β_1- and β_2-adrenoceptors (Katayama et al., 1990).

Therefore, at present, assuming that β-adrenoceptors of the melanophore have discrete affinities for each of the adrenergic agonists used, the Hofstee curves were analysed by making use of the method described by Feldman (1972) and Minneman et al.(1979b).

In each Hofstee curve two asymptotes of the curve were drawn. For example, in the concave-curve for the effect of isoprenaline (Fig 3a), an asymptote of (h) and another one (l) were determined by the parameter-fitting method as was described by Feldman (1972). The asymptote (h) and the other (l) represent a population of β-receptors with a high affinity for isoprenaline and another population with a low affinity for isoprenaline, respectively. The slope of each asymptote represents the affinity constant for the respective populations (Kd). The y-axis-intercepts of the asymptotes (D_l) and (D_2) give the respective populations of β-receptors. The y-axis-intercept of the Hofstee curve (D_3) is the sum of the two y-axis-intercepts of the asymptotes.

In the case of Fig. 3a, β-receptors with a high affinity for isoprenaline were considered to be

β_1-receptors and those with a low affinity for isoprenaline were considered to be β_2-receptors.

The percentages of β_1- and β_2-receptors to the total populaition of β-receptors were 38.9 % and 61.1%, respectively. The affinity constant of isoprenaline for β_1-receptors was 0.59 (nM) and the affinty constant for β_2-receptors was 25 (nM).

The results of the graphical analyses are presented in Table 1. The mean percentages of β_1- and β_2-receptors to the total population of β-receptors were 37.4 ±5.5% and 62.6 ±5.5%, respectively, in the melanophore of *Oryzias latipes*.

(c) Hill plots

Hill plots were obtained from the data presented in the concentration-response curves (Fig. 6).

Hill coefficients which were the slopes of the Hill plots for the effects of the β-agonists used were all less than 1.0 (Table 1).

The values of EC_{50} which was the concentration of the β-agonist that evoked 50% of the maximum response of melanophores were determined on the Hill plots as the conentrations of the β-agonists that yielded $D/Dmax\text{-}D = 1$. The EC_{50} of a β-agonist seems to represent the efficiency of the β-agonist for evoking the dispersion of pigment in the melanophore The order of the efficiency of the β-

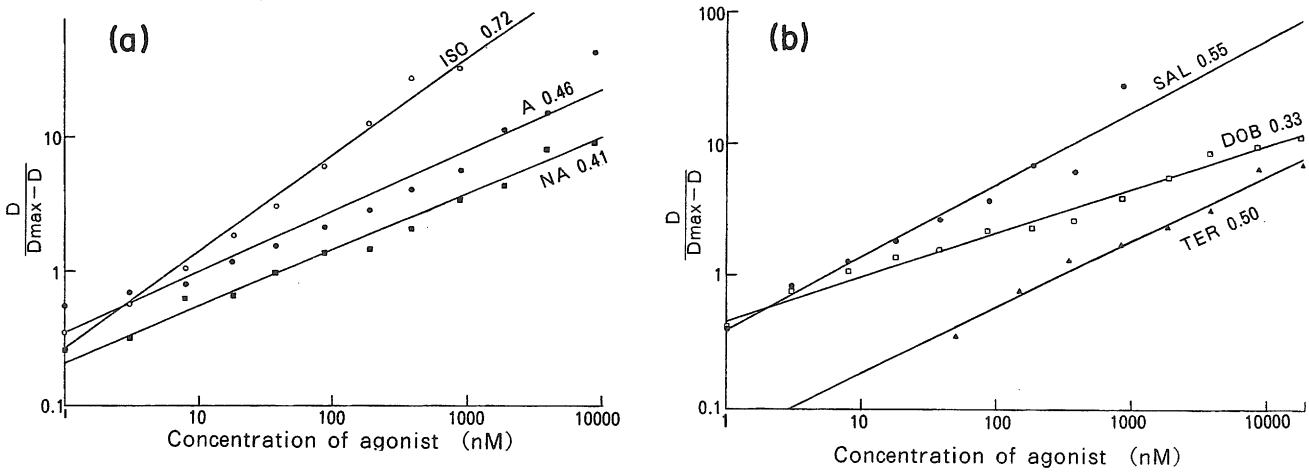

Fig. 6. Hill plots for the effects of the β-agonists on melanophores.The data were transformed from the concentration-response curves shown in Fig. 2. (a): isoprnaline (ISO), adrenaline (A) and noradrenaline (NA). (b): salbutamol (SAL), dobutamine (DOB) and terbutaline (TER). The numbers beside the names of β-agonists are Hill coefficients. Ordinate: D/Dmax-D; D, the mean extent of the pigment-dispersion in the melanophores caused by a given concentation of a β-agonist; Dmax, the maximum extent of the pigment-dispersion caused by the β-agonist.

Table 1. Characteristics of β-adrenoceptors of melanophores of Oryzias latipes estimated by quantitative analyses of the effects of β-adrenergic agonists on the melanophores.

β-adrenergic agonist	specificity	Percentages of β_1 and β_2		Affinity constant (Kd)		EC_{50} (nM)	Hill coefficient
		β_1	β_2	β_1	β_2		
Isoprenaline	β_1 β_2	38.9	61.1	0.59	25	6.3	0.72
Adrenaline	β_1 β_2	47.4	52.6	0.28	119	10.3	0.46
Noradrenaline	β_1	35.8	64.2	0.82	120.5	41.8	0.41
Dobutamine	β_2	37.1	62.9	198	1.05	10.7	0.33
Salbutamol	β_2	36.5	63.5	76.9	1.3	5.7	0.55
Terbutaline	β_2	28.8	71.2	3333.3	84	309.0	0.50
(mean, S.D. = ± 5.5)		37.4	62.6				

agonists used was as follows: salbutamol > isoprenaline > adrenaline > dobutamine > noradrenaline > terbutaline. According to this result, β-adrenoceptors of the melanophore of *Oryzias latipes* belong to the β_2-type.

Discussion

Morishita et al. (1985) reported that β-adrenoceptors of the melanophore of *Oryzias latipes* were β_2-type. They observed that salbutamol and terbutaline which were both β_2-selective agonists inhibited the pigment-aggregating response of denervated melanophores to exogenous noradrenaline, and the inhibitory effects of these β-agonists on the pigment-aggregating response to noradrenaline were abolished by propranolol.

The present experiments revealed that all of the β-agonists used exhibited the pigment-dispersing effects on the melanophores in which pigment had been aggregated by melatonin and the order of the efficiency for the pigment-dispersing action of the β-agonists was salbutamol > isoprenalin > adrenaline > dobutamine > noradrenaline > terbutaline. This order of the efficiency or potency indicates that the β-adrenoceptors of the melanophore of *Oryzias latipes* are β_2-type according to the law of Lands et al. (1967). Thus, the results of our present study agree with the results shown by Morishita et al. (1985).

Katayama et al. (1990) reported that isoprenaline, adrenaline, noradrenaline, salbutamol, dobutamine and terbutaline were all effective in dispersing pigment within melanophores whose pigment had been aggregated by verapamil in the goby, *Tridentiger obscurus*. They considered that the melanophores of the goby possess both β_1- and β_2-adrenoceptors, and estimated that the percentages of β_1- and β_2-receptors for the total population of β-receptors was 18.6% and 81.4% , respectively, from Hofstee analyses of the effects of the β-agonists.

In melanophores of *Oryzias latipes*, it is also possible that β_1- and β_2-adrenoceptors coexist in the membrane, because as the present results indicate, noradrenaline which is a β_1-selective agonist (Lands et al., 1967; Minneman and Molinoff, 1980) and salbutamol which is a β_2-selective agonist (Carlsson et al., 1977; Rugg et al., 1978; Minneman et al., 1979a) are both effective in dispersing pigment in the melanophores.

It was estimated, therefore, after the method described by Katayama et al. (1990), that the percentages of β_1- and β_2-receptors for the total population of β-receptors were 37.4% and 62.6%, respectively.

According to the data shown by Minneman et al. (1979a), Kd values of terbutaline and dobutamine for inhibition of [^{125}I] iodohydroxybenzylpindrol binding to β-adrenoceptors in rat heart and lung suggest that terbutaline and dobutamine are rather specific for β_2-receptors, while isoprenaline and adrenaline are rather specific for β_1-receptors in respect of their Kd values.

In the present Hofstee analyses of the effects of β-agonists, asymptotes of the concave curves representing populations with high affinity for noradrenaline, isoprenaline and adrenaline were considered to be β_1-receptors. By contrast, populations with high affinity for salbutamol and terbutaline were considered to be β_2-receptors. In the case of the Hofstee curve for dobutamine, a population with a high affinity for dobutamine was determnined to be β_2-receptors, although dobutamine had been stated to be a β_1-selective agonist (Stene-Larsen, 1981; Brodde, 1991), for according to Minneman et al. (1979a), dobutamine can be regarded as a non-specific β-agonist and it sometimes exhibits selctivity for β_2-receptors.

The Hofstee curves presented in Figures 3, 4 and 5 can be calssified into three types, namely; (1) noradrenaline and isoprenaline type, (2) salbutamol and terbutaline type and (3) an intermediate type which is adrenaline type. The Hofstee curve for the effect of dobutamine apparently belongs to (2) type. In this respect, dobutamine may well be classified into a β_2-agonist in the present case.

In the present study, analyses of Hofstee plots for the effects of β-adrenergic agonists on the melanophores were made on the assumption that there is two heterogeneous receptors, and no negative cooperativity among β-receptors. However, there is possibility that negative cooperative interactions exist among β-receptors, because Hofstee plots for the effects of isoprenaline and adrenaline which were non-specific agonists (Rugg et al., 1978; Minneman et al., 1979b) were both concave-upwards curves. Moreover, Hill coefficients of the β-agonists used were all below 1.0. It is conceivable, therefore, that β_1- and β_2-adrenoceptors coexisting in the membrane of the melanophore exhibit negative coopertivity when a β-agonist acts on the receptors.

In order to clarify the validity for the assumption that there are two heterogenous β-receptors in the melanophore membrane, and to reveal the existence of negative cooperativity among β-receptors, further investigations on the effects of β-agonists on the melanophore are desirable.

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