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Effects of Divalent Cations on Actions of Informational Molecules on Melanophores of *Zacco temmincki*

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Abstract

Using denervated melanophores in isolated scales of the dark chub *Zacco temmincki*, effects of divalent cations, Ca^{++}, Sr^{++}, Ba^{++}, Mg^{++} and Mn^{++} on responses of the melanophores were investigated.

The Ca^{++} was not required for melanosome aggregation mediated by alpha adrenergic receptors or by melatonin receptors and for melanosome dispersion mediated by beta adrenergic receptors. The Ca^{++} was required for the melanosome-dispersing action of melanophore-stimulating hormone (MSH). Sr^{++}, Ba^{++}, Mg^{++} and Mn^{++} could not replace the Ca^{++}. Mn^{++} reversibly inhibited the action of MSH. These results indicate that the Ca^{++} is the only divalent cation specifically required for MSH action on the melanophores of *Z. temmincki*.

Introduction

In melanophores of *Zacco temmincki*, four kinds of receptors, which take part in melanosome movement within the cells, have been recognized. Those are two types (alpha and beta) of adrenergic, melatonin and melanophore-stimulating hormone (MSH) receptors (Iga, 1980; Iga and Matsuno, 1980). The alpha adrenergic and the melatonin receptors *in vivo* regulate melanosome aggregation in response to norepinephrine, a neurotransmitter released from the chromatic nerve terminals and melatonin, a hormone of the pineal gland, respectively. The beta adrenergic receptors mediate melanosome dispersion through the binding with epinephrine probably secreted from the adrenal gland. The MSH receptors activated by MSH secreted from the pituitary gland control melanosome dispersion.

It has been demonstrated that Ca^{++} is required for the melanosome-dispersing action of MSH on melanophores of the frog (Dikstein et al., 1963; Veerdonk, 1976; Vesely and Hadley, 1976, 1979) and the lizard (Vesely and Hadley, 1971, 1976, 1979) and that Sr^{++} and Ba^{++} can replace the Ca^{++} necessary for the MSH action. Quite recently, Fujii and Miyashita (1980) reported the similar effects of MSH on the catfish, *Parasilurus asotus*.

In the present experiments, we examined 1) whether Ca^{++} was required for MSH action on melanophores of the dark chub *Zacco temmincki*, 2) whether Ca^{++} was also

required for the responses mediated through the other types of receptors, both types of adrenergic and melatonin receptors, 3) whether the Ca^{++} was replaced by other divalent cations.

Materials and Methods

The dark chub, *Zacco temmincki* was used in this study. The melanophores (the second type, Iga and Matsuno, 1980) in the scales isolated from an area within the dark band were used. Denervated melanophores were employed in order to eliminate some possible participations in the nerve function. The denervated melanophores were obtained by intraperitoneal injection of 6-hydroxydopamine (80 μg/g) (Iga and Takabatake, unpublished).

An isolated scale was mounted in a perfusion chamber filled with a physiological solution (128 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM Tris-HCl buffer, pH 7.2). Ca^{++}-free physiological solution was prepared by substituting an isotonic amount of NaCl for $CaCl_2$ in the physiological solution. The drugs used were norepinephrine hydrochloride, isoproterenol hydrochloride, melatonin, alpha melanocyte stimulating hormone (MSH) and 6-hydroxydopamine, which were purchased from Sigma Chemical Co..

The motile response of the melanophores was recorded photoelectrically by a CdS cell and a paper recorder, as described previously (Iga, 1975). The experiments were performed at temperature between 20°C and 25°C.

Results

1. *Effects of Ca^{++} on the alpha adrenergic response*

Norepinephrine induces a rapid aggregation of melanosomes within the melanophores. In the denervated melanophores, the threshold of norepinephrine for the melanosome aggregation was about 5×10^{-10}M and full aggregation was attained at 10^{-8}M.

The degree of melanosome aggregation to 5×10^{-9}M norepinephrine, which induced an intermediate melanosome aggregation, was examined in the physiological solution and in the Ca^{++}-free physiological solution. As is shown in Fig. 1, a degree of aggregation was represented by a percentage of the magnitude of aggregation (a) at 5 min after the application to the complete aggregation (b) induced by 10^{-7}M norepinephrine, that is, $a/b \times 100$.

The result is shown in Table 1. No significant difference was observed between the means.

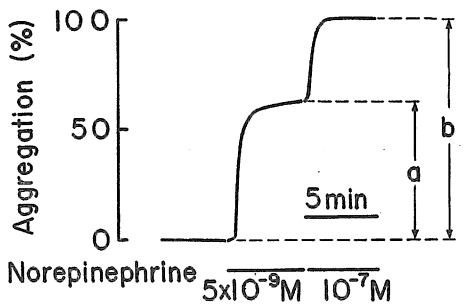

Fig. 1. A typical recording showing melanosome-aggregating effect of norepinephrine (5×10^{-9}M) on *Zacco* melanophores. The effect was expressed by a/b×100, where 'a' was the magnitude of aggregation to 5×10^{-9}M norepinephrine at 5 min after the application and 'b' was that of complete aggregation induced by 10^{-7}M norepinephrine.

Table 1. Melanosome-aggregating effects of norepinephrine and melatonin on *Zacco* melanophores in the presence or absence of Ca^{++} in experimental solution.

Experimental solution	Aggregation (%)	
	Norepinephrine (5×10^{-9}M)	Melatonin (10^{-9}M)
Normal physiological solution	68.0±17.4	23.0±12.0
Ca^{++}-free physiological solution	65.8±22.5	28.5±14.1

Each value represents the mean and the standard deviation of ten measurements.

2. *Effects of Ca^{++} on melatonin-induced melanosome aggregation*

Melatonin was extremely effective in inducing melanosome aggregation (Iga and Matsuno, 1980). Effect of Ca^{++} on the aggregating response of the melanophores to 10^{-9}M melatonin, which induce a moderate melanosome aggregation, was examined. The degree of aggregation was expressed as a percent response, where the degree of aggregation to 10^{-7}M melatonin was assumed as a full aggregation.

The result is shown also in Table 1. The melatonin-induced aggregation was normally induced in the Ca^{++}-free medium.

3. *Effects of divalent cations on MSH-induced melanosome dispersion*

Teleost melanophores in an excised piece of skin maintain the state of melanosome dispersion in physiological solution. Therefore, in order to analyze dispersion response, it is necessary to make the melanosomes keep an aggregating state. In this experiment, 10^{-7}M melatonin was conveniently used for this purpuse, in which the

melanophores maintained fully aggregated.

MSH, when applied in the presence of melatonin, rapidly dispersed the melanosomes which had been aggregated by melatonin (Fig. 2, a). The dispersion induced by MSH was markedly inhibited in the Ca^{++}-free physiological solution (Fig. 2, b; also Fig. 4). Furthermore, addition of 0.5 mM EDTA to the Ca^{++}-free medium completely blocked the melanosome dispersion (Fig. 2, c). These results indicate that the Ca^{++} is obligatory on the action of MSH on *Zacco* melanophores.

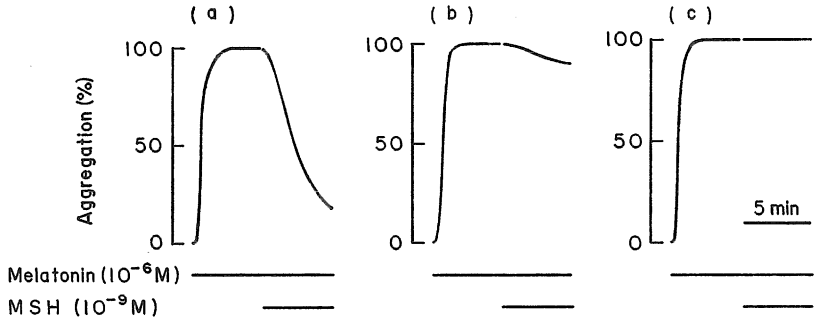

Fig. 2. Responses of *Zacco* melanophores to melatonin and MSH. (a), in the normal physiological solution; (b), in the Ca^{++}-free physiological solution; (c), in the Ca^{++}-free physiological solution containing 0.5 mM EDTA.

Next, we examined whether the Ca^{++} necessary for the MSH action could be replaced by other divalent cations, Sr^{++}, Ba^{++}, Mg^{++} and Mn^{++}. In this experiment, the melanosome aggregation was induced by melatonin dissolved in the Ca^{++}-free physiological solution. A dispersion by MSH (10^{-9}M) at 5 min after the application was expressed by a percentage of a drop (a) induced by MSH from a level of melatonin-

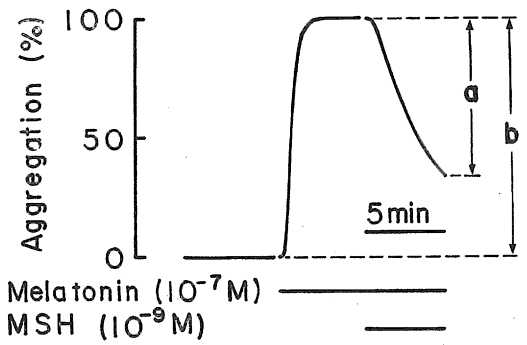

Fig. 3. A typical recording of melanosome-dispersing effect of MSH (10^{-9}M) on *Zacco* melanophores. The effect was indicated by a/b × 100, where 'a' was the magnitude of dispersion induced by 10^{-9}M MSH in the presence of 10^{-7}M melatonin at 5 min after the application, 'b' was that of aggregation to 10^{-7}M melatonin.

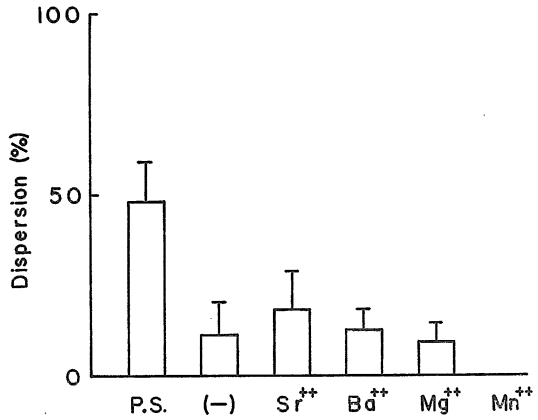

Fig. 4. Melanosome-dispersing effects of MSH on *Zacco* melanophores in various experimental solutions as indicated. P.S., the physiological solution; (−), the Ca^{++}-free physiological solution; Sr^{++}, Ba^{++}, Mg^{++} and Mn^{++}, solutions in which Ca^{++} in the physiological solution was isotonically replaced by one of these cations. Each bar represents the mean±standard deviation of ten measurements.

induced aggregation (b), i.e., $a/b \times 100$. These results are illustrated in Fig. 4. In the Ca^{++}-free physiological solution, the dispersion response was extremely inhibited, as mentioned in the above paragraph. The inhibition of the MSH action in deficiency of Ca^{++} could not be removed by adding Sr^{++}, Ba^{++}, Mg^{++} or Mn^{++}. Mn^{++} appeared to rather inhibit the action of MSH.

To get more precise information on the effects of these divalent cations on the MSH action, the responses of the melanophores to MSH were examined in the phy-

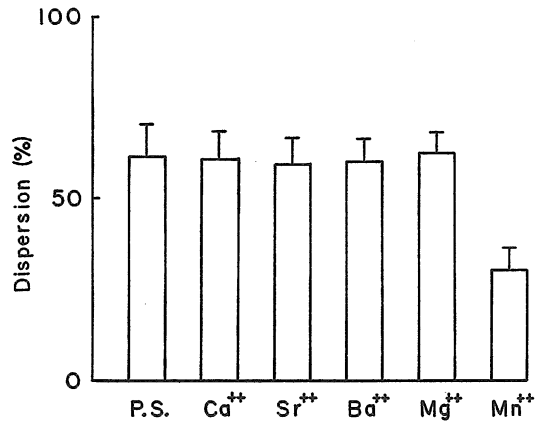

Fig. 5. Melanosome-dispersing effects of MSH on *Zacco* melanophores in various experimental solutions as indicated. P.S., the physiological solution; divalent cations on the abscissa indicate those added in the physiological solution, in which those concentration were 1.8 mM. Each bar represents the mean±standard deviation of ten measurements.

siological solution including those divalent cations. The results are represented in Fig. 5. Sr^{++}, Ba^{++} or Mg^{++} could not affect the degree of the dispersion response induced in the normal physiological solution. This degree remained unaltered also in the physiological solution containing a two-fold concentration of Ca^{++}. On the contrary, in the physiological solution containing Mn^{++}, the MSH action was significantly inhibited. The effect of Mn^{++} was reversible. These results indicate that the Mn^{++} inhibits the melanosome-dispersing action of MSH.

4. *Effects of divalent cations on the beta adrenergic response*

The effects of some divalent cations on the dispersing response of the melanosomes in response to isoproterenol, a rather specific agonist of beta adrenergic receptors, were examined. The experimental procedure was the same as that of MSH, except substituting isoproterenol (10^{-9}M) for MSH.

No significant differences were observed among all the means, in the normal physiological solution, in the Ca^{++}-free physiological solution and in the solution substituted Sr^{++}, Mg^{++} or Mn^{++} for Ca^{++} in the physiological solution (Fig. 6).

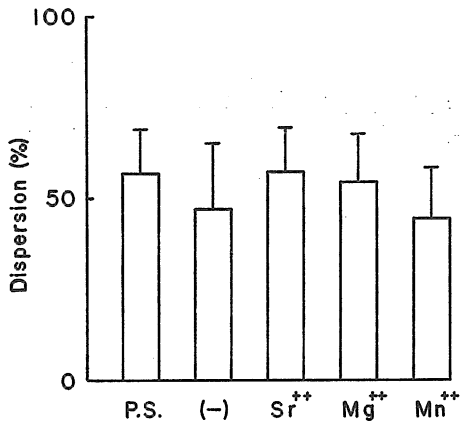

Fig. 6. Melanosome-dispersing effects of 10^{-9}M isoproterenol on *Zacco* melanophores in various experimental solutions as indicated. P.S., the physiological solution; (−), the Ca^{++}-free physiological solution; Sr^{++}, Mg^{++} and Mn^{++}, solutions in which the Ca^{++} in the physiological solution was isotonically replaced by one of these cations. Each bar represents the mean±standard deviation of ten measurements.

Discussion

The preparations for melanophores used in the present experiments, are suitable for pharmacological studies, because the diffusion barrier for chemicals is minimal (Iga and Matsuno, 1980).

The alpha adrenergic response of *Zacco* melanophores was not affected by the

absence of Ca^{++} from the medium as reported in the frog (Vesely and Hadley, 1976, 1979). The Ca^{++} was not obligatory on melanosome aggregation also in response to melatonin. The melatonin-induced response appeared to rather increase in the Ca^{++}-free medium, although no significant difference was observed between the means.

It has been demonstrated that the Ca^{++} was required for the melanosome-dispersing action of MSH on melanophores in the lizard (Vesely and Hadley, 1971, 1976, 1979), in the frog (Dikstein et al., 1963; Veerdonk, 1976; Vesely and Hadley, 1976, 1979) and also in the catfish (Fujii and Miyashita, 1980). The present experiments showed that the Ca^{++} was necessary for the MSH action on *Zacco* melanophores. The role of the Ca^{++}, in the present materials, could not be replaced by Sr^{++}, Ba^{++} and Mg^{++}, in differing with the results of some other investigators that Sr^{++} and Ba^{++} could replace the Ca^{++} (Vesely and Hadley, 1979; Fujii and Miyashita, 1980). This difference is not clear, but it may be pointed out that the scale melanophores of *Z. temmincki* are excellent for analyzing the effects of chemicals on melanophore responses.

In *Zacco* melanophores, two kinds of receptors, which mediate melanosome dispersion, beta adrenergic and MSH receptors, are recognized (Iga, 1980). It has been discussed that melanosome dispersion induced by MSH and by isoproterenol is brought about through activation of adenylate cyclase in the membrane of melanophores, then by an increase of intracellular cAMP (Goldman and Hadley, 1969; Hadley and Goldman, 1969; Abe et al., 1969; Veerdonk, 1976; Geschwind et al., 1977; Miyashita and Fujii, 1980). The Ca^{++} was required for the MSH action, but not for the isoproterenol action. Therefore, the site of action of the Ca^{++} seems to be on an initial step of the receptor response.

Mn^{++} inhibited the melanosome-dispersing action of MSH. Although the inhibition mechanism remained unknown, the inhibition was not due to an oxidizing effect of Mn^{++} from an experiment by using sodium metabisulfate, an oxidation inhibitor. Mn^{++} appears to inhibit an initial step of the MSH action, because melanosome dispersion by isoproterenol is not inhibited by Mn^{++}.

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