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Source: Zoological Science, 13(3) : 351-356

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.351

ZOOLOGICAL SCIENCE **13**: 351–356 (1996)

Signal Transduction of MCH in Melanophores of the Tilapia, *Oreochromis niloticus*

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ABSTRACT—Using split-fin preparations of the tilapia, *Oreochromis niloticus*, the effects of various drugs affecting the signal transduction pathway on MCH-induced aggregation of melanosomes were examined. A phospholipase C inhibitor (neomycin sulphate), protein kinase C (PKC) inhibitors (H-7 and staurosporine), PKC activators (SC-9 and TPA) and calmodulin inhibitors (W-7 and W-5) did not block melanophore response to MCH. However, forskolin, an adenylate cyclase stimulating drug, diminished MCH-induced aggregation of melanosomes in a dose-dependent manner. In fact, intracellular concentration of cyclic AMP (cAMP) in MCH (10 nM)-treated melanophores was found to be about 54% of the control level in melanophores immersed in physiological saline. These results suggest that cAMP is the second messenger for MCH action. Since okadaic acid at 10 μM perfectly inhibited melanosome-aggregating response to MCH, the involvement of protein phosphatase 2B in the response was also implied. In addition, the effects of several drugs on NE-induced aggregation were studied, and the possible signal transduction responsible for melanosome aggregation is comparatively discussed.

INTRODUCTION

In teleost fish, melanin-containing dark pigment cells are called "melanophores". These cells usually exhibit a high motile activity: aggregation or dispersion of pigment granules (melanosomes) in response to neurotransmitters and hormones. Norepinephrine (NE) is a major nervous cue which causes melanosome aggregation. Many researchers reported that melanophore receptors mediating nerve-controlled aggregation of pigment were characterized to be of α_2-adrenoceptor subtype, and that NE-induced aggregation was associated with a reduction of cytosolic content of cyclic AMP (cAMP) (Negishi *et al.*, 1982; Andersson *et al.*, 1984; Morishita, 1987). However, Oshima *et al.* (1990) indicated that NE-induced pigment aggregation was inhibited by W-7, an antagonist of calmodulin, in blue damselfish melanophores. Using fish melanophores loaded with fura 2, a fluorescent Ca^{2+}-indicator, it was also shown that the stimulation of adrenoceptors by NE is accompanied by an increase in intracellular Ca^{2+} level (Oshima *et al.*, 1988; Toyohara and Fujii, 1992) . In addition, Fujii *et al.* (1991) reported that exogenously applied inositol 1,4,5-trisphosphate (IP_3) caused pigment aggregation in detergent-treated melanophores of tilapia. Thus, Ca^{2+} and calmodulin are thought to be also involved in NE-induced aggregation of melanosomes.

As hormonal substances, melanin-concentrating hormone (MCH) and α-melanophore stimulating hormone (α-MSH) are well known. As for the mechanism of action of α-MSH, which induces melanosome dispersion, a considerable amount of evidence has been accumulated (Fujii and Oshima, 1986). However, only a little is known about the mechanism of MCH action because it was recently purified from chum salmon pituitaries (Kawauchi *et al.*, 1983). Working on some fish species, Oshima *et al.* (1985, 1986) have shown that MCH acts directly on pigment cells through its specific receptors on the cell membrane. In the present study, we further examined the effects of various drugs affecting signal transduction pathway on pigment aggregation caused by the peptide in order to facilitate the understanding of transduction mechanism of MCH. In addition, a change in cAMP level within melanophores induced by MCH stimulation was first examined.

MATERIALS AND METHODS

Both sexes of the tilapia, *Oreochromis niloticus*, 10-12 cm in body length, were used. A part of tail fin was excised and split in the physiological saline solution of the following composition (mM): NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5.6, Tris-HCl buffer 5.0 (pH 7.2). The split-fin preparation was fixed on a holder, epidermis side down. The holder was mounted on a perfusion chamber and the entire assembly was put on the stage of a light microscope. The responses of melanophores were recorded by the photoelectric method (Oshima and Fujii, 1984). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5), N-(6-aminohexyl)-5-

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chloro-naphthalenesulfonamide hydrochloride (W-7), N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), 1-[N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) and okadaic acid were obtained from Seikagaku Corporation (Tokyo). Neomycin sulphate (NS, Wako Pure Chemical, Osaka), staurosporine (Sigma Chemical, St. Louis), 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Wako Pure Chemical), forskolin (Wako Pure Chemical) and melanin-concentrating hormone [MCH; purity (HPLC) 98.9%] synthesized at Peptide Institute (Minoh, Osaka) were also used. Stock solution was prepared by dissolving each chemical in distilled water (H-7, W-5, W-7, KN-93, NS, MCH) or DMSO (SC-9, KN-62, TPA, staurosporine, forskolin), and stored at -75°C in the dark. All stock solutions were diluted with physiological saline just prior to use. The final maximum concentration of DMSO in the medium was 1% and below. Norepinephrine (NE) solution was prepared by dilution of an injection fluid of norepinephrine hydrochloride (Sankyo, Tokyo).

After the application of MCH (1 nM) for 3-5 min or NE (50 nM) for 2-3 min, a split-fin preparatione was rinsed with saline until pigment granules within melanophores were dispersed. The preparation was then pretreated with a drug affecting signal transduction pathway for 5 min and exposed to MCH solution for 3-5 min or NE solution for 2-3 min in the continued presence of the drug. After saline rinse for 15-30 min, the recovery from the effects of the drug was confirmed. In the experiments with forskolin, the split-fin preparation was not pretreated with the chemical. In the case of okadaic acid, skin pieces were stimulated by MCH or NE in the absence of okadaic acid after the preincubation with the chemical for 10 or 30 min.

To determine the cytosolic content of cyclic AMP, melanophores were isolated from the skin tissue: scales and small pieces of fins were treated with Ca^{2+}- and Mg^{2+}-free, Dulbecco's phosphate buffered saline (NaCl 136.9 mM, KCl 2.7 mM, $Na_2HPO_4 \cdot 12H_2O$ 8.1 mM, KH_2PO_4 1.5 mM) that contained 0.02% EDTA (Dojindo Laboratories, Kumamoto) for 10 min and then with 0.25% collagenase (type II, Sigma) for 30 min. The suspension of cells was filtered through stainless-steel mesh and centrifuged at 150 g for 10 min. The treatment of the skin tissue with collagenase was repeated five times. The precipitate, i.e., the aggregation of the cells was resuspended in Eagle's minimal essential medium (Nissui Seiyaku, Tokyo) supplemented with 100 U/ml penicillin (Meiji Seika, Tokyo), 100 μg/ml streptomycin (Meiji Seika) and 10% fetal calf serum (GIBCO Laboratories, Grand Island, New York) and inoculated in a culture dish. Three hr later, the culture medium was carefully replaced with fresh medium for removal of non-pigment cells and tissue debris that remained unattached to the substratum. Thus, the concentration of melanophores reached about 80%. The melanophores anchored on the substratum were then dislodged by moderate pipetting in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline, concentrated upon centrifugation and divided into two groups. Melanophores of the first group were treated with MCH (10 nM) for 10 min, and the cells of another group were kept in phosphate buffered saline during this period of time. The melanophores were then frozen in liquid nitrogen until measurements of cAMP content. Subsequent procedures were almost the same as those described by Negishi *et al.* (1982). For the measurements of the concentration of cAMP by radioimmunoassay, Yamasa cyclic AMP assay kit (Yamasa Shoyu, Choshi, Chiba) was used. The protein concentrations were also determined according to the method reported by Lowry *et al.* (1951).

All experiments were performed at room temperature between 23-26°C.

RESULTS

Response of tilapia melanophores to MCH

Figure 1 shows the relationship between the concentration of melanin-concentrating hormone (MCH) and the degree of pigment aggregation. Even at 100 pM, about 67% aggregation occurred, and complete aggregation was observed at 10 nM. At concentrations of 100 nM or more, however, the number of melanophores whose melanosomes were not completely aggregated increased with the increase in concentration of MCH. MCH at 10 μM never induced full aggregation of pigment. That is, MCH at concentrations higher than 100 nM

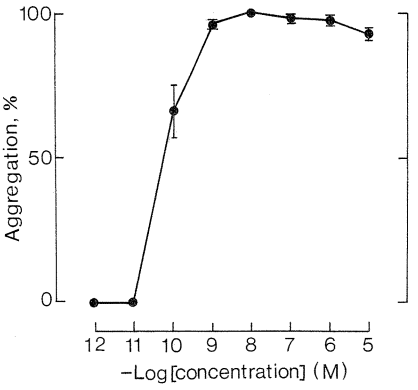

Fig. 1. Relationship between the concentration of melanin-concentrating hormone (MCH) and the magnitude of the pigment-aggregating response of melanophores of the tilapia. After MCH was applied for 6 min, 5 μM norepinephrine was added for 5 min to obtain the maximal (100%) aggregation of melanosomes. Each point represents the mean ± SE of 7 measurements on different animals.

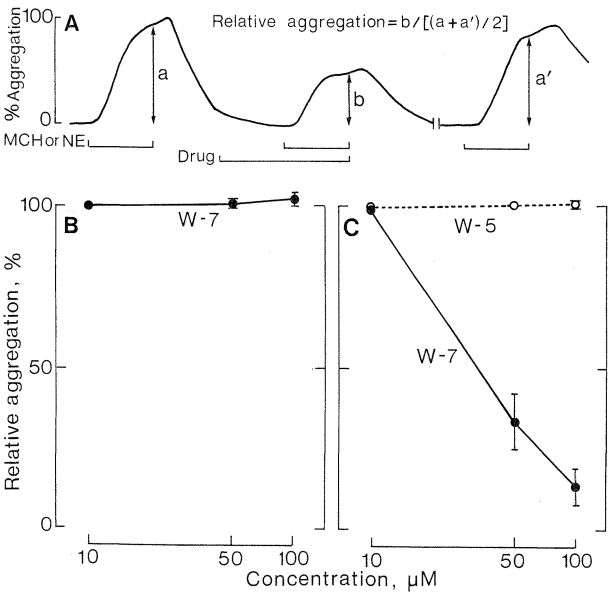

Fig. 2. Relationship between the concentration of W-7 and the extent of melanosome aggregation caused by 1 nM MCH (B) or by 50 nM NE (C). After skin preparations were preincubated with W-7 for 5 min, they were exposed to MCH or NE for 3-5 min or 2-3 min, respectively, in the presence of W-7. No influence of W-5 on NE-induced aggregation was also shown in C. As for relative aggregation (ordinate) in B and C, refer to A. Each point represents the mean ± SE of 6 measurements on different animals.

showed a self-antagonism in tilapia melanophores like in *Synbranchus* melanophores (Castrucci *et al.*, 1987). Therefore, MCH at 1 nM or 10 nM was used in the present experiments.

Effects of reagents affecting DG-IP_3 pathway on MCH action

To determine whether the diacylglycerol-inositol trisphosphate (DG-IP_3) pathway is involved in MCH-induced aggregation of melanosomes, several drugs that affect serial steps of DG-IP_3 pathway were used. In these experiments, MCH solution at 1 nM was employed.

A phospholipase C inhibitor, neomycin sulphate (1, 10 and 50 μM), and protein kinase C (PKC) inhibitors, H-7 (10, 50 and 100 μM) and staurosporine (1, 10 and 100 nM), did not diminish MCH-induced aggregation of melanosomes. SC-9 (1 and 5 μM), a PKC activator, also had no effect on melanophore response caused by MCH. A phorbol ester, TPA, which mimicks the action of DG and activates PKC, did not affect melanophore response to MCH at 10, 50 and 100 nM.

To determine the possible involvement of Ca^{2+}/calmodulin complex in MCH-induced aggregation of melanosomes, the influence of W-7, a calmodulin antagonist, was studied. The chemical never arrested the aggregation of melanosomes even at 100 μM, as shown in Figs. 2 and 3.

Comparatively, the effects of H-7 and W-7 on norepinephrine (NE; 50 nM)-induced aggregation were examined. H-7 never blocked melanosome-aggregating response to NE, whereas W-7 inhibited the response in a dose-related manner (Fig. 2). The inhibitory action of W-7 was reversible (Fig. 3). There was practically no effect of W-5 on melanosome aggregation caused by NE. In a few skin pieces, W-7 and W-5 at concentrations higher than 50 μM induced a slight level of aggregation *per se*.

Inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II, KN-62 and KN-93, were also found to have no effect on pigment aggregation caused by MCH and NE.

These results suggest that neither DG nor IP_3 is related to MCH-induced aggregation of pigment, and that it is IP_3 and not DG that is involved in aggregation caused by NE. A participation of calmodulin kinase II in NE-induced aggregation was also denied.

Effect of activator of adenylate cyclase on melanophore response

When MCH (1 nM) was applied to the skin preparation in the presence of forskolin (10, 50, 100 nM and 1 μM), an adenylate cyclase (AC) stimulating agent, pigment aggregation was depressed in dependence of concentration of forskolin (Figs. 4 and 5). However, NE-induced response was not affected by the drug at 10, 50, and 100 nM. At 1 μM, forskolin inhibited the aggregation to some extent. Time course of melanophore response to NE in the presence of 1 μM forskolin is typically shown in Fig. 5.

From the results, an important role of AC in MCH-induced aggregation of pigment is suggested. In NE-induced aggregation, AC may participate in part.

Effect of okadaic acid on pigment aggregation

Okadaic acid (OA) isolated from the marine sponge, *Halichondria okadai*, is a specific inhibitor of protein phosphatase 1 (PP1) (IC_{50}=315 nM), phosphatase 2A (PP2A) (IC_{50}=1.2 nM) and phosphatase 2B (PP2B) (IC_{50}=4.5 μM), whereas it has little effect on protein phosphatase 2C (PP2C) (Bialojan and Takai, 1988).

After almost complete aggregation of melanosomes in response to 1 nM MCH (control aggregation) was attained, the split-fin preparation of tilapia was rinsed with saline until melanosomes were redispersed. The preparation was then incubated with OA at 10 μM for 10 or 30 min and subsequently stimulated by 1 nM MCH. The split preparation of tail fin excised from one and the same individual was incubated in physiological saline containing 1% DMSO for 10 or 30 min in control experiments. MCH-induced aggregation of melanosomes was perfectly inhibited by OA (10 μM) treatement for 30 min, although the preincubation with 1% DMSO for the same period of time never affected MCH action (Fig. 6). Melanophores did not recover from the effect of OA even after they were rinsed with saline for 30 min. Ten-min incubation with OA did not block melanophore response to MCH.

The effect of OA (10 μM) on NE (50 nM)-induced aggregation was comparatively studied. In three out of five measurements, pretreatment with OA for 10 min reduced the extent of aggregation: 55.6%, 67.7% and 76.3% of control aggregation. When melanophores were incubated for 30 min, their response to NE was completely arrested like the case of MCH.

Cytosolic content of cAMP in MCH-treated melanophores

The cAMP levels within tilapia melanophores immersed in saline (control) and in MCH solution (10 nM) for 10 min were determined. In three individuals, the mean value of cAMP for MCH-treated cells was 8.6 ±1.2 pM/mg protein, about 54% of the control level (15.8 ±1.8 pM/mg protein). The difference was significant (P< 0.05, Studen's t test). Therefore, cAMP is surely the second messenger for MCH action in tilapia melanophores.

DISCUSSION

Recently, Abrão *et al.* (1991b) reported that the dose-response curves for MCH were shifted to the right in skins treated with drugs affecting DG-IP_3 pathway, suggesting that protein kinase C (PKC) might mediate the pigment-aggregating activity of MCH in teleost (*Synbranchus marmoratus*) melanophores. However, the results in this study demonstrate that, in the tilapia, PKC may not participate in the intracellular events caused by MCH, nor by NE, since neither the action of MCH nor that of NE was affected by inhibitors and activators of the enzyme. Pigment aggregation by MCH in tilapia melanophores is triggered by a decrease in cyclic AMP (cAMP) level via inactivation of adenylate cyclase (AC): forskolin, an AC activator, perfectly arrested the action of MCH (1 nM) at 1 μM, and a 46% reduction of the content of cytosolic cAMP

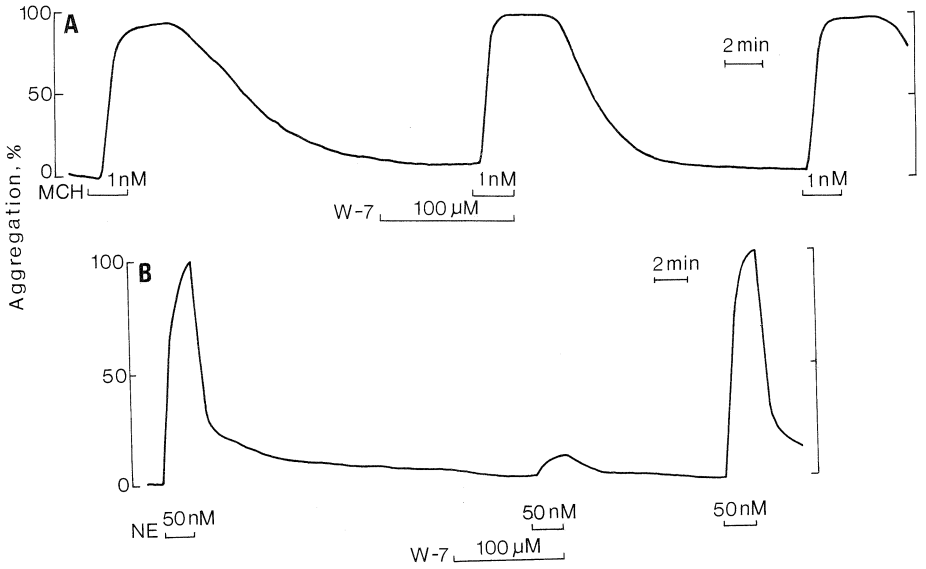

Fig. 3. Typical recordings showing the effects of W-7 on melanosome aggregation caused by MCH (A) or NE (B). W-7 (100 μM) inhibited only NE action.

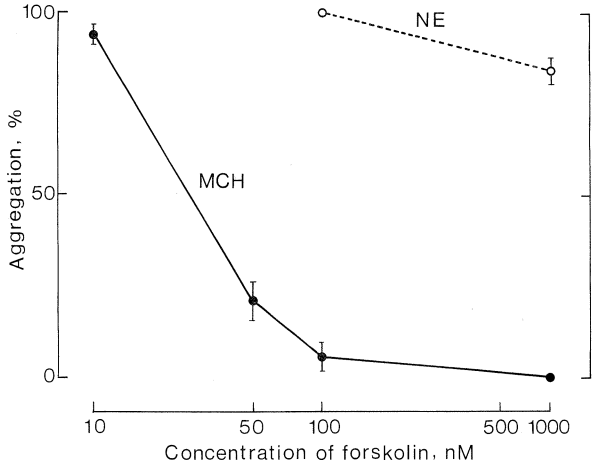

Fig. 4. Relationship between the extent of melanosome aggregation and the concentration of forskolin, a stimulator of adenylate cyclase (AC), contained in MCH (1 nM) and NE (50 nM) solutions. MCH or NE was applied for 3-5 min or 2-3 min, respectively, in the presence of forskolin. As for relative aggregation (ordinate), refer to Fig. 2A. Each point represents the mean ± SE of 6 measurements on different animals.

was produced at MCH concentration of 10 nM. In melanophores of *Corydoras paleatus*, we also observed almost complete inhibition of MCH action by 1 μM forskolin (unpublished data).

In NE-induced aggregation, the situation was more complicated compared with tha case of MCH. Since W-7 inhibited NE action in a dose dependent manner, it seems to be beyond doubt that IP_3, Ca^{2+} and calmodulin are related to the response. However, forskolin at 1 μM, by which MCH action was perfectly arrested, also inhibited NE-induced aggregation to some extent. The present observation with a microscope showed that when NE solution containing forskolin (1 μM) was applied to melanophores, pigment granules were once aggregated up to the fairly high level and subsequently redispersed. The progress of the dispersion came to a stop shortly and the intermediate level of aggregation was kept up until the initiation of saline rinse (see Fig. 5).

In melanophores of *Corydoras paleatus*, whose pigment granules aggregate more slowly than those in tilapia melanophores in response to NE, the action of NE was also inhibited by W-7 and forskolin (unpublished data). Unlike the case of tilapia melanophores, the extent of inhibition by forskolin was higher than by W-7. Therefore, it is likely that Ca^{2+} is responsible for such rapid aggregation response as is observed in tilapia melanophores, while cAMP mainly participates in gradual aggregation of melanosomes, the case in *Corydoras* melanophores. Thus, in NE-induced aggregation of pigment, the reduction of intracellular cAMP content may also be a signal in addition to the increase in Ca^{2+}.

In the present report, however, it should be emphasized

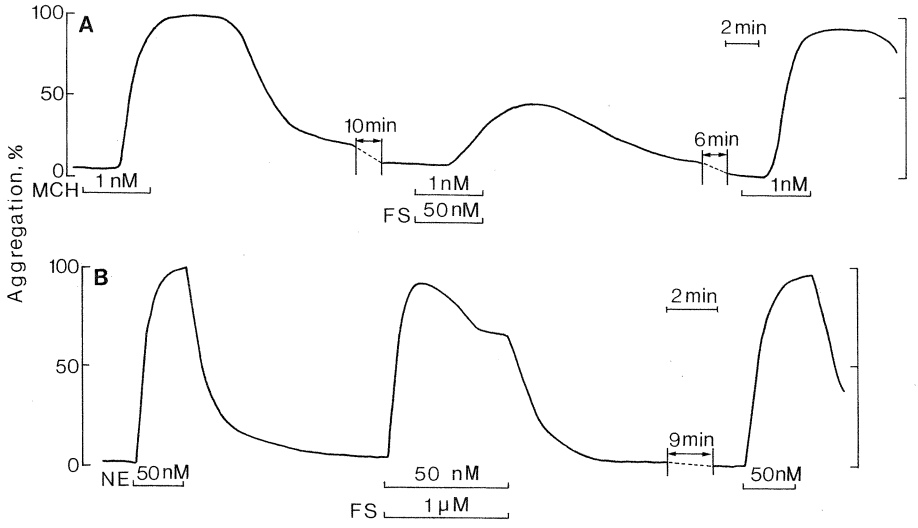

Fig. 5. Typical recordings showing the effects of forskolin (50 nM or 1 μM) on melanosome aggregation caused by MCH (A) or NE (B). Although forskolin inhibited both MCH and NE actions, the former action was more effectively blocked.

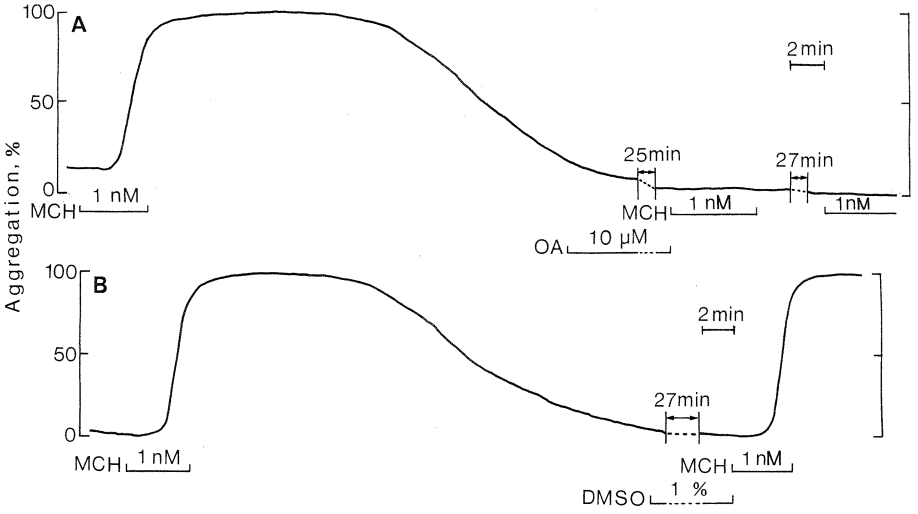

Fig. 6. The inhibitory effect of okadaic acid (OA) on MCH (1 nM)-induced aggregation. Treatment of melanophores with OA (10 μM) for 30 min completely inhibited aggregation (A), but the cells incubated with 1% DMSO for 30 min responded again to MCH (1 nM) by full aggregation of pigment (B). In both recordings, split preparations of tail fin excised from one and the same tilapia were used.

that α_1-adrenoceptors coupled with phospholipase-activating G protein also play a very important role in NE-induced aggregation, although several researchers came to the conclusion that α_2-adrenoceptor system regulates teleost melanophore responses on the basis of analyses with α_1 and α_2 adrenergic agonists and antagonists (Andersson *et al.*, 1984; Morishita 1987; Abrão *et al.*, 1991a).

Using medaka melanophores permiabilized by digitonin treatment, Nagamori *et al.* (1994) demonstrated that exogenously applied cAMP caused pigment dispersion in the presence of ATP after NE-induced aggregation. The dispersion was inhibited by H-89, a specific inhibitor of PKA. Further, the involvement of phosphorylation of a 57kD protein in pigment dispersion was suggested by Rozdzial and Haimo (1986) in tilapia melanophores. That is, an activation of PKA by cAMP may bring about protein phosphorylation, leading to melanosome dispersion. Conversely, a decrease in cytosolic concentration of cAMP by MCH via a decrease in AC activity may lead to sharp reduction of PKA activity. Thus, the activity of PKA might comparatively fall below protein phosphatase (PP) activity, resulting in dephosphorylation of a 57kD protein, which, in turn, might induce aggregation of pigment.

In the present experiments, we confirmed the inhibitory effects of okadaic acid (OA; 10 μM), a PP inhibitor, on MCH and NE actions. Judging from the effective concentration of OA, the possible involvement of protein phosphatase 2B (PP2B; calcineurin) is implied. Thaler and Haimo (1990) first indicated an important role of PP2B in epinephrine-induced pigment aggregation in melanophores of the tilapia, *Oreochromis mossambicus*. We also describe here the

inhibitory effect of OA on MCH-induced aggregation, suggesting that, even if a decrease in cytosolic content of cAMP brought about by MCH lowers the activity of PKA, melanosome aggregation never occurs without the activity of PP2B. Thus, pigment translocation within melanophores seems to be regulated by both activities of PKA and PP2B. On the other hand, pigment-aggregating actions of MCH and NE may not involve a protein phosphorylation by Ca^{2+}/calmodulin kinase II, since KN-62 and KN-93, inhibitors of the kinase II, did not affect melanophore responses caused by MCH and NE.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Reaerch from the Ministry of Education, Science, Sports and Culture, Japan to N. Oshima (No. 04640674).

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(Received February 7, 1996 / Accepted March 26, 1996)