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# Possible Involvement of Nitric Oxide in Signaling Pigment Dispersion in Teleostean Melanophores

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**ABSTRACT**—The possible involvement of nitric oxide (NO) in regulating the motile activities of teleostean melanophores was studied in the dark chub *Zacco temmincki* (Cyprinidae, Cypriniformes) and in the translucent glass catfish *Kryptopterus bicirrhis* (Siluridae, Siluriformes). NO donors, including (±)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexaneamide (NOR 1), molsidomine (MSD), sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), had no pigment-aggregating action on melanophores, but actively dispersed melanosomes in those cells. Among those reagents, NOR 1, a spontaneous releaser of NO, was the most effective. Inhibitors for nitric oxide synthase (NOS), i.e. *N*ω-nitro-L-arginine methyl ester (L-NNA), *N*ω-nitro-L-arginine (L-NAME) and *N*ω-monomethyl-L-arginine (L-NMMA), showed melanosome-aggregating effects. A membrane-permeable analogue of cyclic guanosine-3',5'-monophosphate (8-Br-cGMP) was effective in dispersing melanosomes. The sum of these results suggests that NO plays an active role in the elaborate control of color changes in teleosts by dispersing pigment in melanophores via activation of soluble guanylyl cyclase to increase cytosolic levels of cGMP.

**Key words:** coloration, nitric oxide, melanophore, pigment dispersion

## INTRODUCTION

Integumentary colorations of animals depend primarily on the distribution and activities of pigment cells in the skin. In poikilothermal animals, such cells are called chromatophores and are very often responsible for dynamic color changes of skin, which are of course important for survival. These changes in color result from the motile activities of the chromatophores, and the regulation of motile responses of chromatophores has been examined especially well in teleosts. It is now known that the chromatophores are neurally regulated by the sympathetic nervous system, and also by several hormonal substances (cf. Fujii, 1993, 2000; Fujii and Oshima, 1986, 1994). In addition, we have recently reported that chromatophore motility is also modulated by a paracrine factor, endothelin (Fujii *et al*., 1993; Hayashi *et al*., 1996; Fujita and Fujii, 1997; Murata and Fujii, 2000). Although many factors have already been shown to be involved in the regulation of chromatophores, we have always been assuming that there may be others, since changes in the hues and patterns that exist among these weak animals are so remarkable and elaborate.

In 1980, Furchgott and Zawadzki demonstrated that relaxation of isolated mammalian vascular smooth muscle in response to acetylcholine (and to some other substances) depended on the presence of an endothelial layer. They presented evidence that the effect was mediated by a short-lived humoral substance which diffuses to neighboring smooth muscle cells. Later, this endothelium-derived relaxing factor (EDRF) was shown to be nitric oxide (NO) (Palmer *et al*., 1987; Ignarro *et al*., 1987). NO is an unstable gaseous radical, that is highly reactive. Although first reported as a unique vasodilator released from endothelial cells, it has now been shown to have various effects on different biological processes in a number of animal species, including invertebrates. Nowadays, much attention has been paid to the role of NO in neural transmission in the brain as well as in the peripheral nervous system, in addition to its function in the cardiovascular systems (Moncada *et al*., 1991; Kiss, 2000). Moreover, current studies have indicated that the most important physiological process of NO in intracellular signaling process may be the activation of soluble guanylyl cyclase. That enzyme promotes the formation of cyclic guanosine monophosphate (cGMP), which results in the phosphorylation of certain proteins more directly related to the motor functions of the cells (Waldman and Murad, 1987; Moncada *et al*., 1991).

Based on their results that an inhibitor of NO synthesis, *N*ω-nitro-L-arginine methyl ester (L-NAME) reduced the mel-

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atonin-induced aggregation of melanosomes, Nilsson *et al.* (2000) recently reported that NO may play a role in the aggregation of pigment in melanophores of the African clawed toad, *Xenopus laevis*. Using teleostean species, we have also examined the possible role of NO in signaling the motile responses of chromatophores. The results we have obtained to date indicate that NO may take part in dispersing melanosomes, at least in melanophores of teleosts. Our results are in contrast to those obtained with the amphibian species in terms of the direction of the pigment displacement. Brief accounts of this work have been presented elsewhere (Fujii and Hayashi, 1996; Fujii, 2000).

## MATERIALS AND METHODS

### Materials

Adult specimens of the dark chub *Zacco temmincki* (Cyprinidae, Cypriniformes) and the translucent glass catfish *Kryptopterus bicirrhis* (Siluridae, Siluriformes) of both sexes were employed in this study. They were purchased from local dealers in Tokyo and in the Chiba Prefecture.

### Isolation of skin preparation

Skin specimens were prepared in a physiological saline solution for teleosts, which had the following composition in 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).

When the dark chub was used, scales were plucked from the longitudinal stripe running along the middle part of the trunk. In most teleostean species, chromatophores on the scales are present in the dermis of the skin that covers the bony scale. In this material, the chromatophores are present in the dermis on the inner surface of the scale. Without an epidermis, which constitutes a very strong diffusion barrier, chromatophores are much more easily accessible by chemicals and drugs than are those of many other species (Iga and Matsuno, 1980; Hayashi and Fujii, 1993). Namely, the scales of this species could conveniently be employed for detecting the effects of chemical stimuli on motile responses of chromatophores. The method for setting scales on the microscopic stage for measurement has been described elsewhere (Hayashi and Fujii, 1993).

We occasionally employed translucent glass catfish that lack scales. In such cases, the skin preparations were excised from a narrow zone along the dorsal median line. Detailed procedures for preparing the specimens and setting them to measure melanophore responses were the same as those described in a previous paper (Hayashi and Fujii, 1994).

When a skin piece from a common fish is equilibrated in standard physiological saline, melanosomes in the melanophores usually become dispersed. However, in the case of translucent glass catfish, they tend to aggregate gradually into the perikarya of the cells. Therefore, in order to precisely assess the pigment-aggregating action of a drug, the melanosomes must be dispersed beforehand. For this purpose, α-melanophore-stimulating hormone (α-MSH; Sigma Chemical, St. Louis, MO) was employed at a very low concentration (100 pM), and was added both to the standard physiological saline and to other experimental media (Fujii and Miyashita, 1982; Hayashi and Fujii, 1994).

### Nervous stimulation

In some experiments, a $K^+$-rich saline solution was employed to stimulate nerves, because the elevation of $K^+$ concentration in the extracellular space is known to act as a sympathetic stimulant via release of catecholaminergic neurotransmitter in common teleosts (Fujii, 1959, 1993; Fujii and Oshima, 1994) or of acetylcholine in silurid catfishes, including the translucent glass catfish (Kasukawa and Fujii, 1984). In the present experiments, saline containing 50 mM $K^+$ ions was used for this purpose, and the $Na^+$ concentration described for the standard saline were compensatorily decreased so that the final osmolarity remained constant.

In some experiments, skin pieces were stimulated in a field of sine-wave alternating current generated by a CR oscillator (AG-203, Kenwood, Tokyo). It is known that such an electrical field stimulates sympathetic fibers to liberate neurotransmitters (Fujii and Novales, 1968). The stimulating waves were monitored by a storage oscilloscope (5111A, Tektronix, Beaverton, OR).

### Measurement of motile responses of melanophores

The photoelectric method for measuring motile responses of melanophores was identical to that described recently (Fujii *et al.*, 2000). In the present study, the response of a single melanophore was determined by restricting the area to be measured to a circular with a diameter of 150 μm.

In the photoelectric recordings shown, the magnitude of the melanosome-aggregating response was expressed as a percentage of the full response, taking the fully dispersed state as zero, and with the maximal aggregation as 100%. When we used dark chubs, the latter was usually attained during application of 2.5 μM norepinephrine hydrochloride (NE; racemic modification; Sankyo, Tokyo) for more than 5 min. The concentration of NE in this study is expressed in terms of the concentration of the biologically active L-(–)-isomer. In melanophores of the glass catfish, the maximal pigment aggregation (100%) was reached by application of 50 μM acetylcholine chloride (ACh; Daiichi Seiyaku, Tokyo), because peripheral transmission to the melanophores has been disclosed to be cholinergic. In fact, melanophores of this and other catfish species belonging to the family Siluridae surprisingly lack α-adrenoceptors, and muscarinic cholinoceptors take part in the nervous control in their place, although the innervation is sympathetic postganglionic as usual (Fujii *et al.*, 1982; Fujii, 2000).

The ratio of the breadth (abscissa: time) and the length (ordinate: magnitude of response) of the desirable part of an original recording always varied. In order to exhibit the responses more plausibly therefore, the ratios of the records selected for publication were converted to be about 1 : 5. The procedures for such conversion were practically identical to those described elsewhere (Murata and Fujii, 2000).

All physiological and pharmacological measurements were performed at room temperature between 20 and 25°C.

### Chemical denervation of melanophores

In order to exclude the possible involvement of adrenergic neurotransmitter in the process of NO action, responses of denervated melanophores were sometimes examined. Chemical sympathectomy was performed by a single intraperitoneal injection of 6-hydroxydopamine (6-OHDA; Sigma Chemical, St. Louis, MO) to a dark chub at a dosage of 80 μg/g body weight (Iga and Takabatake, 1982). About 24 hr after the injection, the fish was utilized for study. Scales plucked from the sympathectomized fish were first examined as to whether the melanophores had been successfully denervated, using the $K^+$-rich saline described above. If melanophores did not respond by aggregating their melanosomes, they were regarded to be denervated (Fujii, 1959).

### Drugs used

In addition to α-MSH, NE, ACh and 6-OHDA, melanin-concentrating hormone (MCH, Peninsula Lab., Belmont, CA) was conveniently employed as an agent to aggregate pigment in melanophores.

As NO donors, the following chemicals were used: (±)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexaneamide (NOR 1; Dojindo Lab., Kumamoto), molsidomine (MSD; Biomol Res. Lab., Plymouth Meeting, PA), sodium nitroprusside (SNP; Sigma Chemi-

cal, St. Louis, MO) and glyceryl trinitrate (GTN, Nippon Kayaku, Tokyo). NOR 1 was dissolved in 100% dimethyl sulfoxide (DMSO) to make a 10 mM stock solution, and was prepared fresh daily. The stock solution of 10 mM MSD was prepared by dissolving it in 10% ethanol, and that of 100 mM SNP was dissolved in distilled water. GTN solution was obtained in ampoules for clinical use.

Inhibitors for nitric oxide synthase (NOS) used were *N*ω-monomethyl-L-arginine acetate (L-NMMA), *N*ω-nitro-L-arginine (L-NNA) and *N*ω-nitro-L-arginine methyl ester (L-NAME); all were the products of Sigma Chemical. Guanosine 3',5'-cyclic monophosphate (cGMP; Na salt) and its 8-Br analogue (8-bromoguanosine 3',5'-cyclic monophosphate, 8-Br-cGMP; Na salt) were also manufactured by Sigma Chemical.

## RESULTS

### Pigment-aggregating effects of NO donors

In order to characterize the possible involvement of NO in the control of chromatophore motility, we first examined whether NO donors elicited pigment aggregation in melanophores of dark chubs and/or translucent glass catfish. We tested 4 NO donors, namely NOR 1, MSD, GTN and SNP, applying them one at a time to skin preparations for more than 15 min. None of the NO donors caused any pigment-aggregating action on melanophores of both species of fish within the wide range of concentration from 10 pM up to 100 μM. Using the extremely high dosage of 1 mM, we confirmed that in melanophores of dark chubs, MSD did not aggregate melanosomes. However, at 1 mM, SNP induced a gradual aggregation of melanosomes within 5 min of application, and that aggregation sometimes reached a level of about 80% of the full response attainable by NE. The reaction was quickly reversed to the initial pigment-dispersed state after the removal of the drug. The response was reproducible with repeated application of SNP. Tests at the high concentration of 1 mM for the other two NO donors NOR 1 and GTN, could not be performed because of their lower solubility.

Treatment of skin specimens with those NO donors had practically no impairing effect on the responsiveness of the melanophores themselves: after such treatment, they responded quite normally to other pigment-aggregating agents, such as NE and MCH.

### Pigment-dispersing effect of NO donors

We then examined the pigment-dispersing effects of those

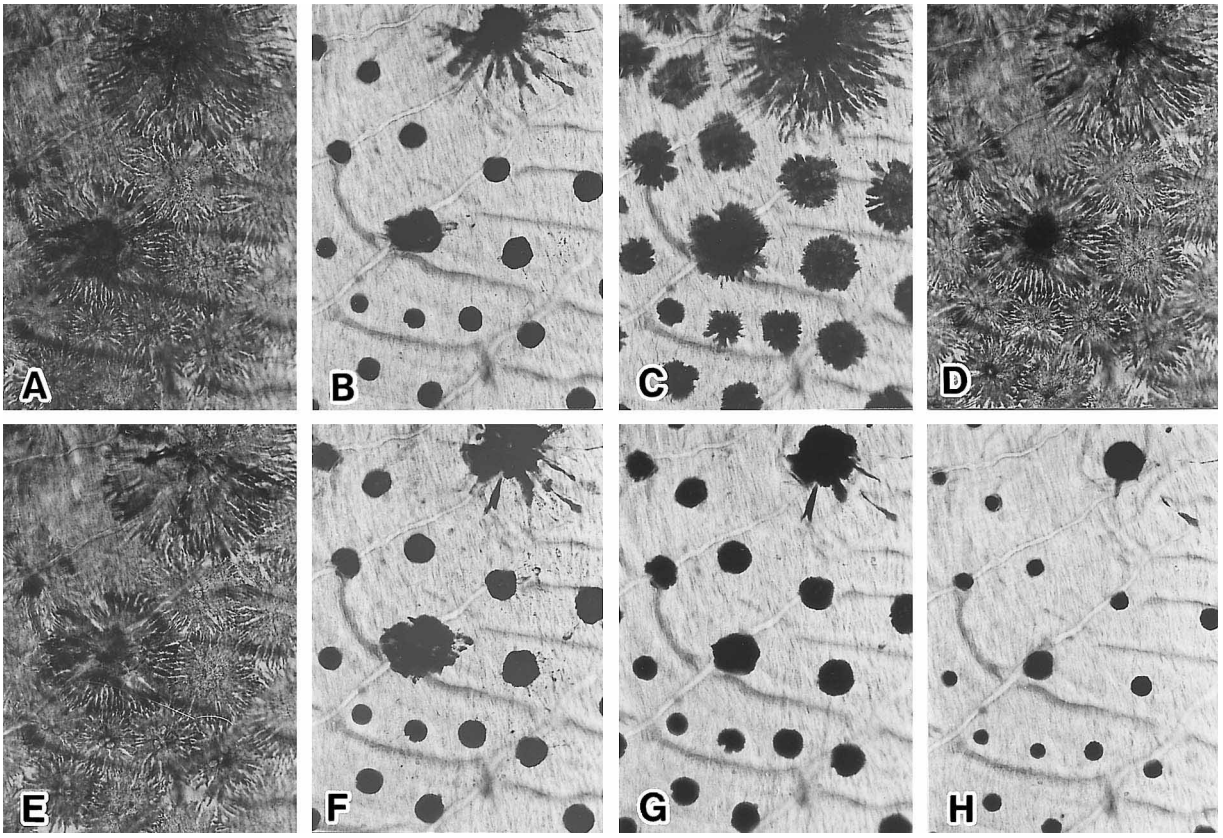


**Fig. 1.** Serial photomicrographs showing the motile response of melanophores on a scale of the dark chub *Zacco temmincki*. **A**: Equilibrated in physiological saline. Melanosomes are completely dispersed within the cells. **B**: 3 min after the application of 200 pM MCH. Melanosomes are almost fully aggregated into the perikarya. **C**: 3 min after the application of 10 μM NOR 1, a spontaneous donor of NO, in the presence of 200 pM MCH. Melanosomes became gradually dispersed by the action of NOR 1. **D**: 3 min after changing the medium to 10 μM NOR 1 without MCH; melanosomes became almost fully dispersed. **E**: State after thorough washing the chemicals with the saline; melanosomes were completely dispersed again. **F**, and **G**: 3, and 10 min after the application of 200 pM MCH, respectively; melanosomes became aggregated, and the state lasted so long as the peptide was present in the medium. **H**: 5 min after the application of 2.5 μM NE; melanosomes were completely aggregated in the perikarya. X110.

NO donors on melanophores of the two species of fish. When equilibrated in physiological saline or in saline with α-MSH, melanophores in isolated skin specimens commonly assume a pigment-dispersed state. To evaluate the pigment-dispersing effect of a certain stimulus, melanosomes in the cells had to be aggregated beforehand. We sought an appropriate pigment-aggregating agent that could induce a sufficient and prolonged aggregation of the pigment as long as it was present in the bathing medium, and we found that MCH was the most suitable agent for that purpose. At very low concentrations, namely, around 100 pM, MCH induced the remarkable aggregation of melanosomes in melanophores of both dark chubs and of translucent glass catfish, a state that persisted even after its removal from the medium. Using that peptide, we examined the possible pigment-dispersing effects of NO donors that are known as "nitrovasodilators" in the medical fields.

Among the four NO donors employed, NOR 1, which elicits spontaneous release of NO, and MSD, an NO donor known to release the radical metabolically, were found to be very

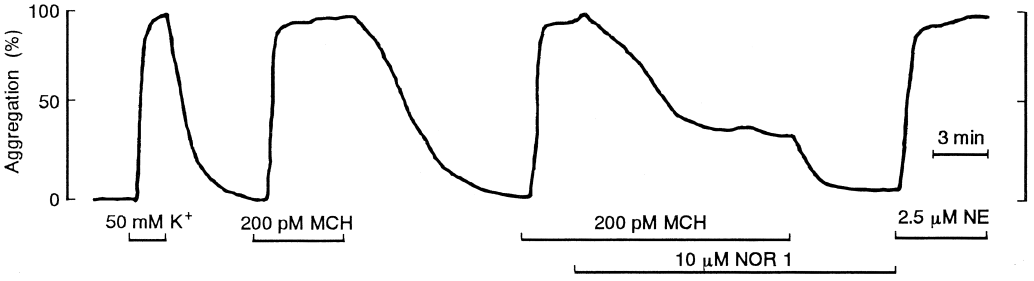


**Fig. 2.** Typical photoelectric recording showing the responses of a single dark chub melanophore to an NO donor, NOR 1. At first, $K^+$-rich saline ($K^+$: 50 mM) was applied for a short time to confirm the normal responsiveness of the cell, then 200 pM MCH was applied. Almost full aggregation of melanosomes was induced, and the state lasted so long as the peptide was present in the medium. After a while, 200 pM MCH was applied again, and NOR 1 was added during the response to MCH. Even in the presence of MCH, the NO donor actively dispersed melanosomes. MCH was then withdrawn, and the melanosome dispersion was accelerated, reaching the fully dispersed level quickly. In the final part of the recording, 2.5 μM NE was applied, which aggregated the pigment almost completely. Abscissa, time. The scale is indicated as a horizontal bar on the right of the panel. Ordinate, magnitude of response as a percentage of the maximal level of pigment aggregation. The explanations regarding the abscissa and ordinate also applies to the figures that follow.

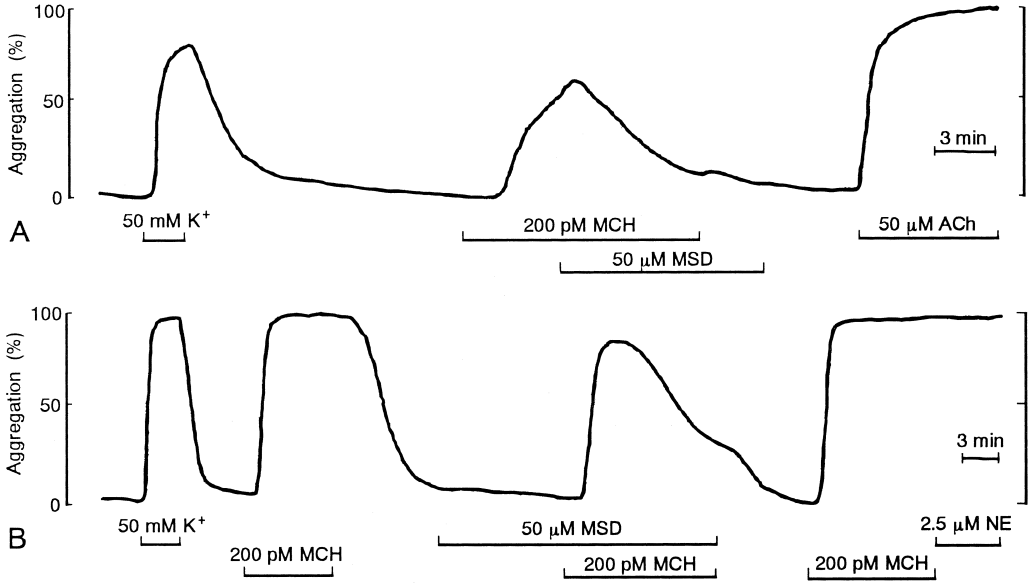


**Fig. 3.** Typical recordings of the responses of individual melanophores of the translucent glass catfish *Kryptopterus bicirrhis* (**A**), and the dark chub *Zacco temmincki* (**B**) to an NO donor, MSD. In both recordings, normal responsiveness of the cell was first confirmed by a brief application of $K^+$-rich saline. **A**: During the course of the pigment aggregation in response to 200 pM MCH, 50 μM MSD was applied. Even under the coexistence of MCH, a remarkable and active dispersion of melanosomes in response to MSD was induced. Finally, 50 μM ACh was applied to the cholinergically controlled cell to induce full aggregation of melanosomes. **B**: After confirming that the *Zacco* cell was normally responsive to MCH, 50 μM MSD was applied, and the melanosomes remained dispersed. Under the coexistence of 50 μM MSD, 200 pM MCH was added. The pigment-aggregating action of MCH was suppressed and reversed gradually. To induce a complete aggregation of pigment, NE was finally administered to the adrenergically controlled cell.

effective in inducing pigment dispersion in melanophores of dark chubs and of glass catfish. Fig. 1 shows a typical series of photomicrographs in which the effect of NOR 1 on melanophores of a dark chub was examined. Melanosomes in the normal dispersed state (A) were first aggregated by 200 pM MCH (B). While the medium still contained the same concentration of MCH, we applied the NO donor (at 10 μM) to the melanophores. Counteracting the action of MCH, it actively dispersed the pigment within 3 min (C). The perfusing medium was then changed to the saline containing NOR 1 but not MCH. Maximal dispersion of melanosomes promptly developed within another 3 min (D). By contrast, MCH alone was sufficiently to aggregate melanosomes (F), and the state was kept even after 10 min (G).

NOR 1 showed such pigment-dispersion action even at relatively low concentrations. In melanophores in which pigmentary organelles had been aggregated by 200 pM MCH, 100 nM NOR 1 induced a moderate pigment dispersion, approximately 50% of the full response. When 10 μM NOR 1 was applied in the presence of MCH at the same strength, it frequently brought about almost a full response, although the extent of the response attained varied considerably. Fig. 2 is a photoelectric recording selected from those in which responses of melanophores of the dark chub were examined.

The pigment dispersing action on melanophores was also confirmed with MSD, and the typical recordings obtained are presented in Fig. 3. Namely, we were able to detect an apparent melanosome dispersion elicited by MSD in both species. In the glass catfish melanophore, in which melanosomes had been previously aggregated by 200 pM MCH, 50 μM MSD actively dispersed them even in the continued presence of MCH (Fig. 3A). In panel B, in which a melanophore of a dark chub was recorded, the action of 200 pM MCH was reduced and gradually reversed under the coexistence of 50 μM MSD.

We also examined the possible pigment-dispersing effects of the other NO donors, namely GTN and SNP, and the photoelectric traces exhibited in Figs 4 and 5 show representative results. As seen in these figures however, the effects of GTN and SNP were not as remarkable as compared with NOR 1 and MSD.

The potencies of NO donors to induce pigment dispersion were considerably different among the four agents employed. In addition, the extent of the pigment dispersion elicited by agents at the same strength varied considerably among preparations. However, we may safely say that their action is concentration-dependent.

**Response of denervated melanophores to NO donors**

The action of NO donors on chemically sympathectomized melanophores was then studied. The responses of denervated melanophores of the dark chub to NO donors were practically the same as those of normally innervated cells: Both NOR 1

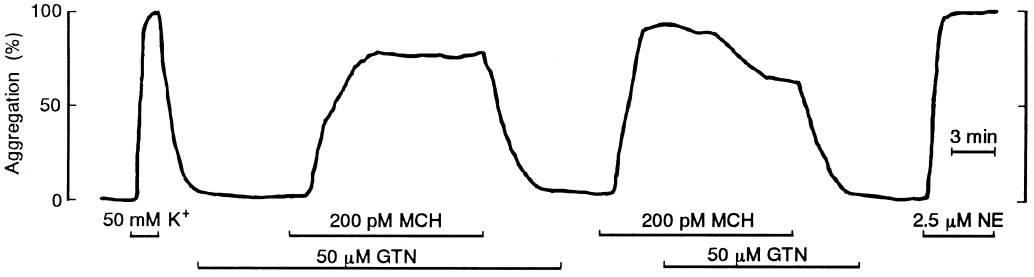


**Fig. 4.** Typical recording showing the effects of an indirect NO donor, GTN, on a single dark chub melanophore. The rapid melanosome aggregation induced by 50 mM $K^+$ indicates the normal responsiveness of the cell. After pretreatment with 50 μM GTN, 200 pM MCH was added to the medium. The induced response in the presence of GTN was recognizably smaller, if compared with the case when MCH alone was applied. We can then see that the MCH-induced aggregation of melanosomes was actively reversed by GTN. When MCH was withdrawn, a rapid dispersion of pigment took place, indicating the active pigment-dispersing effect of GTN.

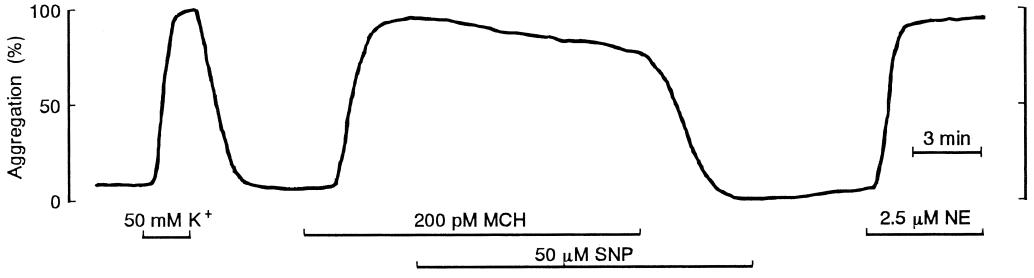


**Fig. 5.** Typical recording showing the effect of an NO donor, SNP, on the pigment-aggregating action of MCH using a single dark chub melanophore. After confirming the normal responsiveness of the cell by $K^+$, 200 pM MCH was applied to induce full aggregation of pigment. When the maximal level was attained, 50 μM SNP was added, which showed a recognizable reversing effect on the action of MCH. When MCH was withdrawn, the rapid dispersion of melanosomes occurred due to the action of SNP.

and MSD were very effective. GTN showed moderate effectiveness, while SNP had only a slight effect even at very high concentrations. As representative experiments, the responses of a denervated melanophore of the dark chub to MSD are shown in Fig. 6A and B. In both recordings, the refractoriness of the melanophore to $K^+$-rich saline ($K^+$: 50 mM) was first displayed. This confirmed that the cell had been successfully deprived of the nervous supply. Under the influence of MSD, the action of MCH was decreased (A), and a gradual dispersion of pigment took place even in the presence of MCH (B).

**Effects of NOS inhibitors on melanophores**

Inhibitors of NOS were also studied for their effects. These included L-NMMA, L-NNA, and L-NAME. It was quite difficult to detect the influences of these agents on the state of melanophores. In some measurements however, we could detect discernible effects. Namely, in melanophores of the glass catfish, the dispersion of melanosomes following their aggregation by electrical field stimulation was often reversed in the presence of one of these agents. Fig. 7 representatively illustrates such a recording, in which L-NNA was used.

**Effects of cyclic GMP on melanophores**

The possible effects of cGMP on melanophores of these fish were then examined. First, we applied cGMP to melanophores in which melanosomes were dispersed, and realized that the nucleotide was entirely ineffective in aggregating the pigment. We then employed a cGMP analogue, i.e. 8-Br-

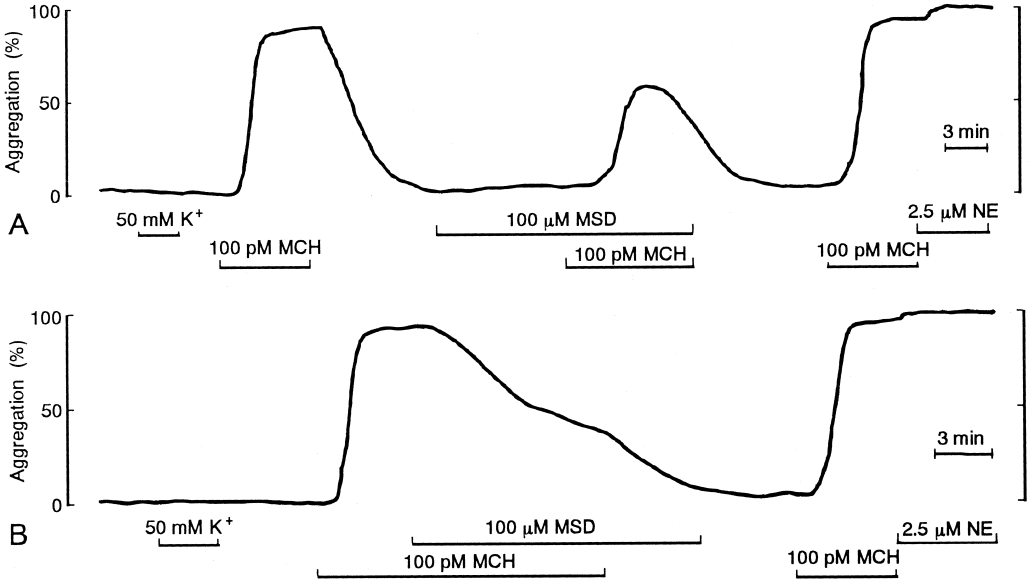


**Fig. 6.** Typical recordings showing the effects of MSD on the responses of individual denervated melanophores of the dark chub. In both recordings, the lack of responsiveness to $K^+$-rich saline was shown to indicate the successful denervation of the cell. **A**: After confirming the normal responsiveness to MCH, the cell was treated with 100 μM MSD, and then MCH was added. The pigment aggregation aroused was much smaller than that recorded before. Without MSD, the response to MCH was completely restored. Full aggregation of pigment was finally aroused by 2.5 μM NE. **B**: Upon addition of MSD, the pigment aggregation induced by MCH was gradually reversed, and then MCH was withdrawn from the medium. An apparent acceleration of pigment dispersion was observed. After checking the action of MCH without MSD, NE was applied to induce the full aggregation of pigment.

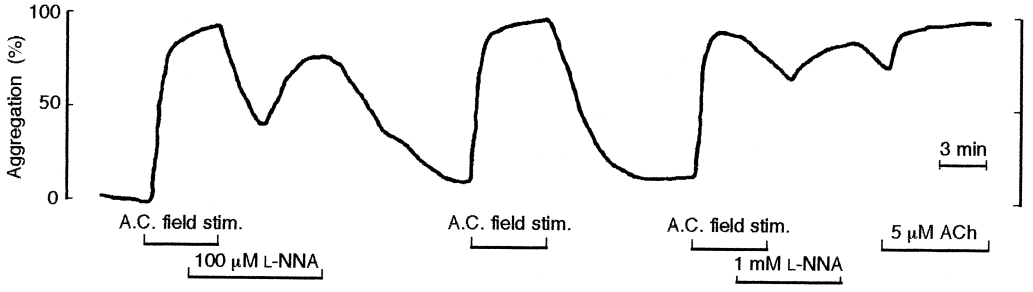


**Fig. 7.** Typical recording showing the effect of an NOS inhibitor, L-NNA, on a single melanophore of a translucent glass catfish. Electrical field stimulation was applied by sine wave AC (10 Hz, 6.3 V/cm) to elicit melanosome aggregation effectively. Upon cessation of the stimulation, a prompt pigment dispersion can be observed (middle part). Although fairly high concentrations were needed, L-NNA gradually reversed the melanosome dispersion following the electrical stimulation (left and right parts). Finally, 5 μM ACh was applied to induce full aggregation of melanosomes.

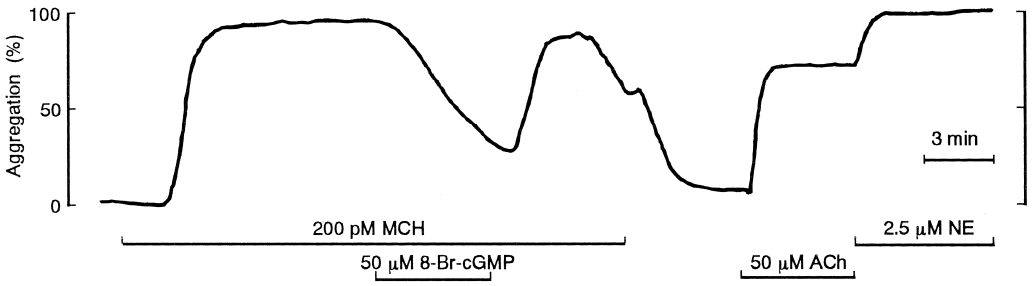


**Fig. 8.** Typical recording showing the effect of 8-Br-cGMP on a single dark chub melanophore. During the melanosome aggregation induced by 200 pM MCH, 50 μM 8-Br-cGMP was applied which aroused a remarkable dispersion of melanosomes. Upon withdrawal of the nucleotide analogue, the melanosomes reaggregated. ACh induced a moderate aggregation of melanosomes, indicating that the cell under study possessed cholinoceptors in addition to the common α-adrenoceptors. Finally, NE was applied to induce maximal pigment aggregation.

cGMP, which is known to permeate cells more readily, and that was again found to be ineffective on melanophores.

We then tried to detect the possible action of cGMP to disperse melanosomes. Again, MCH was employed to aggregate melanosomes beforehand, but cGMP was ineffective in subsequently dispersing them. We then tried 8-Br-cGMP, and found that in some cases this permeable analogue dispersed the pigment in both species of fish examined. Fig. 8 shows a typical recording in which the responses of a melanophore from a dark chub were examined.

## DISCUSSION

Since its initial demonstration as a novel vasodilating principle in mammals, NO has been shown to be involved in many physiological processes such as anti-platelet aggregation, cytotoxicity of macrophages and neutrophils, and neural transmission. In spite of its extremely short lifetime, this simple gaseous radical has thus established itself as a very common physiological mediator in a number of tissues in vertebrates and in invertebrates. In the present work, we have tried to examine the possible involvement of NO in the control of the motile activity of melanophores, using two teleostean species.

When examining the effects of NO on certain tissues or cells, direct application of a solution containing NO would seem to be the easiest approach, but since it is such an unstable gas, it is practically impossible to prepare such NO solutions. Therefore, several types of NO donors which release NO in the experimental system are widely employed. We also used such reagents, and found that they did not elicit aggregation of melanosomes in melanophores, but actively dispersed them. Both normally innervated and denervated melanophores responded to NO donors similarly. These observations indicate that the mode of action of NO on these cells is common, and that the radical acts directly on the melanophores, resulting in the melanosome dispersion. We then examined the effects of NOS inhibitors, and found that they had a melanosome-aggregating action. Further, a membrane-permeable analogue of cGMP (8-Br-cGMP) was effective in dispersing melanosomes. The sum of these results indicates that NO may play a role in controlling body color changes, at least in some teleosts, by dispersing pigment in melanophores via activation of soluble guanylyl cyclase that increases cytosolic level of cGMP. Although that pathway might be subordinate to the established cAMP and $Ca^{2+}$ signaling systems in chromatophores (Fujii, 1993), NO may function in the delicate and subtle changes of integumantary hues and/or patterns.

The extent of the pigment dispersion induced varied depending on the type of NO donors used and also among preparations. Among those NO donors, NOR 1, which was recently developed, was the most effective and elicited remarkable dispersion of melanosomes even at concentrations as low as 100 nM. Without requiring any chemical modifications or any co-factors, NOR 1 releases NO radicals quite spontaneously in the experimental system, and the amount of NO released is more abundant compared with other NO donors. Thus, the present results on fish melanophores seem to be quite plausible. The release of NO from other types of NO donors is not spontaneous. For example, NO release from GTN is known to require the active participation of a thiol radical (-SH group) *in vivo*. In contrast, the process of NO production from MSD is quite different from that of the other NO donors. At first, MSD is enzymatically converted to SIN-1 (3-morpholinosydononimine), which then releases NO spontaneously. Without participation of a sulfhydryl group, SNP releases NO radicals exclusively by chemical reaction. However, the amount of NO released is known to be relatively small. Furthermore, SNP has a risk of producing cyanogen ($CN^-$). Thus, we think that GTN and SNP may not be suitable for future analyses on the role of NO in the chromatic system of fish.

NO is produced during the enzymatic conversion of L-arginine to L-citrulline by NOS, and the terminal guanidino nitrogen of L-arginine and oxygen are the actual sources of the radical. Although unstable, the NO produced immediately reacts with many substances that play roles in various cellular reactions. Among the substances produced in this cascade, some may have certain physiological activities. When NO donors are experimentally utilized as the source of NO, by-product(s) of the chemical compounds might also influence the cellular responses. For example, an active metabolite of MSD and SIN-1 yields peroxynitrite ($ONOO^-$) as the final prod-

uct. It may naturally be thought that peroxynitrite has an action different from NO. Therefore, although melanophores were shown to respond to NO donors by pigment dispersion, further detailed analyses of the mode of action are needed.

Actually, a number of speculations have been proposed to date about the mechanism of action of NO, and recent assumptions include the ideas that it may act to activate cycloxygenase (Salvemini *et al.*, 1993), reacts with superoxide anions (Mathesis *et al.*, 1992), functions as a metal complex by binding with heme proteins (Ribeiro *et al.*, 1993), or ADP-ribosylate proteins such as the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Brüne *et al.*, 1993; Zhang and Snyder, 1993). It may now be said however, that the most important and plausible action of NO is the activation of soluble guanylyl cyclase (Niroomand *et al.*, 1989).

Recently, Oberg *et al.* (1999) presented preliminary results on an amphibian species, the African clawed toad *Xenopus laevis*, and they reported that NO production in melanophores is required for the functional centripetal movement of melanosomes, but that the effect of NO is not mediated through the increased intracellular concentration of cGMP. Working on the same amphibian species, Nilsson *et al.* (2000) further noted that an inhibitor of NO synthesis, L-NAME, reduced the melatonin-induced aggregation of melanosomes. They concluded that NO may activate soluble guanylyl cyclase, which would lead to increased production of cGMP and activation of cGMP-dependent kinases, resulting in the aggregation of melanosomes. Interestingly, the direction of the NO-induced pigment displacement in *Xenopus* melanophores is opposite to that detected in the present study on teleostean melanophores. Since amphibian melanophores are known to possess considerably different physiological properties from those of teleostean ones, such a difference may be understandable (cf. Fujii, 2000).

In smooth muscle cells of mammals, the muscarinic cholinoceptors-mediated response (either the contraction or the relaxation) is now considered to be due to the activation of guanylyl cyclase. At least in the melanophores endowed with muscarinic cholinoceptors therefore, cGMP can rather easily be assumed to signal the process. Contrary to the established role of cAMP as the intracellular mediator in signaling pigment dispersion in chromatophores (Fujii, 1993; 2000), little information is available about the role played by cGMP: Working on the black goldfish *Carassius auratus*, Abramowitz and Chavin (1974) reported that not only cAMP, but also cGMP or cyclic cytidine 3', 5'-monophosphate (cCMP), induced melanosome dispersion although the potencies of the latter two were lower. On the basis of those results, they proposed the multinucleotide regulation of melanosome dispersion in fish melanophores. Negishi and Obika (1980) have further shown that the dibutylyl analogue of cGMP was more effective than cAMP in dispersing pigmentary organelles in *Oryzias* melanophores and xanthophores, while the light-scattering organelles in leucophores were effectively dispersed by cAMP. From these results, they concluded that cGMP might be the intracellular messenger, rather than cAMP, for signaling centrifugal movements of melanosomes and xanthosomes.

In the present study on the possible role of NO in the control of melanophore motility, we employed two species of fish, i.e. the dark chub and the translucent glass catfish. The former belongs to Cyprinidae, Cypriniformes, while the glass catfish belongs to Siluridae, Siluriformes. Therefore, these two species are phylogenetically rather remote from each other. Based on the results obtained here, it may rather easily be assumed that NO takes part in signaling the motile activities of melanophores, at least in the superorder Ostariophysi to which both orders belong. Further comparative surveys are naturally needed to know whether the NO radical regulates melanophores more generally. As for the action of NO on chromatophores other than melanophores, we have no information and studies should therefore be extended to other types of chromatophores.

Melanophores of the translucent glass catfish have been shown to possess muscarinic cholinoceptors for sympathetic peripheral transmission, replacing ordinary $\alpha$-adrenoceptors (Fujii *et al.*, 1982). Later, we further disclosed that melanophores of fish belonging to various classes within the order Siluriformes possess cholinoceptors (Kasukawa *et al.*, 1986; Fujii, 2000; Fujii and Oshima, 1986). The dark chub, another species used in the present study, does not belong to the catfish family, but rather a member of Cyprinidae, Cypriniformes. Working on this and a closely related cyprinid species, we have recently shown that some, but not all, melanophores possess muscarinic cholinoceptors that mediate pigment aggregation (Hayashi and Fujii, 1993). In the present study, we elucidated the possible role played by NO, and found further that a membrane-permeable analogue of cGMP, 8-Br-cGMP, induced the dispersion of melanosomes in two fish species. In these melanophores therefore, the intracellular signaling role of cGMP may easily be assumed. As of this time however, we have not been able to confirm as to whether the involvement of cGMP in regulating motile responses is restricted only to melanophores that possess muscarinic receptors or whether the involvement is more generally applicable. Along with the role of guanylyl cyclase in the NO system, further detailed analyses need to be performed.

The site of NO generation that influences the motile responses of melanophores has not yet been studied. Current biochemical studies in other systems indicate that NO is synthesized by the action of NOS. In the pigmentary system too, NOS may be involved somewhere in the vicinity of the effector cells (the chromatophores). At present, we presume that endothelial cells of the blood capillaries that run in the vicinity of melanophores are one possible source, as in the case of a paracrine factor, endothelin, that strongly induces melanosome aggregation (Fujii, 2000). The freely permeable NO radical may penetrate melanophores and activate cytosolic guanylyl cyclase, finally eliciting the dispersion of melanosomes.

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