ZOOLOGICAL SCIENCE 19: 829–839 (2002)

An Increase in Extracellular Ca^{2+} Concentration Induces Pigment Aggregation in Teleostean Melanophores

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ABSTRACT—An increase in the concentration of Ca^{2+} ions in the external medium ($[Ca^{2+}]_o$) induced pigment aggregation in melanophores of three species of freshwater teleosts examined. Denervated melanophores were refractory to elevations of $[Ca^{2+}]_o$. The pigment-aggregating action was inhibited by the sympathetic blocking agents, phentolamine, prazosin and yohimbine. Bretylium, an agent known to block the release of the neurotransmitter, interfered with the response effectively. Ca^{2+} blockers, such as Mn^{2+}, verapamil and gallopamil, also inhibited the response, possibly by inhibiting Ca^{2+} entry into the presynaptic elements of melanosome-aggregating fibers. The conclusion is that the increase in $[Ca^{2+}]_o$ may induce membrane depolarization of presynaptic nervous elements around the melanophores, which open the voltage-dependent Ca^{2+} channels there. The liberation of adrenergic neurotransmitter follows, which induces the aggregation of pigment in melanophores.

Key words: melanophores, calcium, calcium blockers, innervation, voltage-dependent Ca^{2+} channel

INTRODUCTION

In addition to their primary role of revealing integumentary colors, chromatophores of many species of animals including fish take dynamic part in the changes of these colors and patterns (Fujii, 1993). Being ascribable to their cellular motility, such changes are believed to be of utmost importance for the survival of individual animals and also of the species. In fish, most chromatophores are dendritic cells, and a number of processes emanate from their cell bodies parallel to the plane of the skin. Pigmentary organelles (termed chromatosomes) migrate centripetally into the perikarya (aggregation), or centrifugally (dispersion) in response to various signals, mostly brought about by sympathetic fibers and/or blood-borne hormones (Fujii, 1993; Fujii and Oshima, 1994). When chromatosomes disperse within the cells, the skin exhibits the color of the chromatosomes more strongly, while it fades when the organelles aggregate into the perikarya.

Since Spaeth (1913) initially made good use of isolated scales from the killifish, *Fundulus heteroclitus*, a number of workers have employed excised skin pieces to investigate the motile mechanisms of chromatophores as well as the mechanisms regulating their motility. Actually, a vast number of reports have appeared on the effects of various substances, including biogenic hormonal and neuronal principles and also of synthetic chemicals and drugs (Fujii, 1993, 2000a). In contrast, the effects of changing ionic environments around the chromatophores have been infrequently investigated (Spaeth, 1913, 1916; Yamamoto, 1933; Kamada and Kinosita, 1944; Kinosita, 1963; Fujii, 1959; Iwata *et al.*, 1959a). As for the influence of Ca^{2+}, reports have also been meager (Spaeth, 1916; Yamamoto, 1933; Kamada and Kinosita, 1944), and up to the present time no sound explanations have been put forward about its effects or its mechanism of action, although the intracellular role of Ca^{2+} has sometimes been examined in some teleostean species (Luby-Phelps and Porter, 1982; Negishi and Obika, 1985; Oshima *et al.*, 1988, 1998; Toyohara and Fujii, 1992; Kots and McNiven, 1994). While working on melanophores of some teleostean species, we have recently succeeded in clearly recording the pigment-aggregating effect of increasing the extracellular Ca^{2+} concentration, and we have tried to analyze its mechanism of action.

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MATERIALS AND METHODS

Materials

The materials used were the dark chub (*Zacco temmincki*), the

Table 1. Concentrations of $CaCl_2$ and NaCl in experimental solutions, in which $[Ca^{2+}]$ was variously increased (mM)

$CaCl_2$	1.8	20.0	30.0	40.0	50.0	60.0	70.0	85.3
NaCl	125.3	98.0	83.0	68.0	53.0	38.0	23.0	0.0

Other components were common, namely (in mM): KCl 2.7, $MgCl_2$ 1.8, D-glucose 5.6, Tris-HCl buffer, 5.0 (pH 7.2).

zebrafish (*Brachydanio wrier*) and the medaka (*Oryzias letups*). Adult specimens, irrespective of sex, were obtained from local dealers in Tokyo or in the Chiba Prefecture. Prior to use, they were maintained in a freshwater aquarium for at least one week for acclimatization.

Melanophores in the scale were examined. In dark chubs, the scales were plucked from the longitudinal dark stripe along the middle of the trunk, while in zebra fish, those from the dark stripes on the dorso-lateral surface were employed. In medakas, scales of wild-type individuals were taken from the anterior dorso-lateral dark surface. The isolated scales were immediately immersed in a physiological saline solution 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, and Tris-HCl buffer 5.0 (pH 7.2). Unless otherwise stated, freshly excised scales were employed.

Each scale was affixed to a cover slip by means of a fine glass needle that had been glued onto the surface of the slip at both ends. Melanophores in the scale of medakas reside in the dermis between the epidermis and the bony scale. Under the binocular dissecting microscope therefore, each scale was held between the glass needle and the cover slip with its bony scale side in contact with the cover slip. In dark chubs and zebra fish, melanophores are present in the thin dermal connective tissue below the bony scale (Iga and Matsuno 1980; Fujii *et al.*, 1993; Hayashi and Fujii, 1993). Therefore, each of those scales was held with the epidermal side in contact with the surface of the cover slip. The cover slip with a scale on its underside was then mounted in a perfusion chamber, and then transferred onto the microscope stage. While the scale was irrigated with various experimental solutions, the responses of the melanophores were observed.

Recording of melanophore responses

Physiological and pharmacological techniques employed were basically the same as those described elsewhere (Fujii *et al.*, 2000). In the present study, the transmission of light through a circular area of skin 150 μm in diameter was measured. That size is sufficient to encircle the domain of a single melanophore in the skin of these species. In order to eliminate possible influences of motile responses of neighboring xanthophores and/or erythrophores, an orange-red plastic filter (SC-56, Fuji Photo Film, Tokyo) was used. A square piece of this filter (12×12 mm) was placed just under the photosensor inside the photographic column of the trinocular system.

The ratio of the breadth (abscissa: time) and the length (ordinate: magnitude of response) of the desirable part of each 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 7:1. The procedure for the conversion was similar to that described elsewhere (Murata and Fujii, 2000).

Method of denervation

Denervation of melanophores was performed by keeping isolated scales in physiological saline overnight (Iwata *et al*, 1959b), and scales left in saline for 20 to 30 hr were employed. To test whether the melanophores were actually denervated, a K^+-rich saline was employed, because denervated cells do not respond to a heightened K^+ by the aggregation of pigment (Fuji, 1959). In the present study, a K^+-rich solution having the following recipe was employed: (in mM): NaCl 78.0, KCl 50.0, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-glucose 5.6, and Tris-HCl buffer 5.0 (pH 7.2). If the melanophore was refractory to the heightened K^+, it was regarded to be denervated.

Ca^{2+}-rich saline

Saline solutions in which the concentration of Ca^{2+} ions was varied were used. In order to keep the osmolarity of the solution to be the same as primary physiological saline, these solutions were prepared by replacing equimolar amounts of Na^+ ions from the normal saline, while the concentrations of $MgCl_2$, D-glucose and the pH buffer were kept constant (Table 1).

Electrical stimulation of chromatic nerves

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 using a storage oscilloscope (5111A, Tektronix, Beaverton, OR).

Drugs used

Autonomic drugs used for pharmacological studies included a sympathetic stimulant, norepinephrine hydrochloride (NE; racemic modification; Sankyo, Tokyo), acetylcholine chloride (ACh; Daiichi Seiyaku, Tokyo), and three α-adrenolytic agents, phentolamine mesylate (Ciba-Geigy, Basel), prazosin and yohimbine (Sigma Chemical, St. Louis, MO). The concentration of NE used to stimulate these receptors was expressed in terms of the concentration of the L-(-)-isomer.

As a substance known to interfere with the release of neurotransmitters from adrenergic postganglionic fibers, bretylium tosylate (Sigma Chemical, St. Louis, MO) was employed. Ca^{2+}-channel blockers employed were $MnCl_2$ (Wako Pure Chemical Ind., Osaka), verapamil (Sigma Chemical, St. Louis, MO), gallopamil (methoxyverapamil; Sigma Chemical, St. Louis, MO), and a recently developed N type channel specific agent, ω-conotoxin GVIA (Sigma Chemical, St. Louis, MO).

All experiments were performed at room temperature between 20 and 27°C.

Computer graphics analysis

Simulation analyses of the effects of changes in the ionic environment on the equilibration potential across the presynaptic membrane were performed using computer graphics, where changes in the equilibration potential are expressed as functions of the extracellular concentrations of various cations and of permeable coefficients assumed for Ca^{2+}. The program employed was the same one developed by one of us (RF) that has been employed in previous studies (e.g., Fujii *et al*, 1991). The data were processed by drafting software, and were appropriately labeled for presentation.

RESULTS

Effects of $[Ca^{2+}]_o$ elevation

As in many other teleostean species, equilibration in normal physiological saline of a scale from the dark chub, the zebrafish or the medaka, brought about the dispersion of melanosomes within the melanophores. Such a typical response is seen in serial photomicrographs shown in Fig. 1 where a scale from a dark chub was employed. When the

concentration of Ca^{2+} was raised to 70 mM in isotonic saline the melanosomes aggregated fairly rapidly (panels A, B, and C). However, in contrast to the effect of NE, this effect was always transient, and the redispersion of pigment can be distinctly seen while the scale was still in the Ca^{2+}-rich saline (panels D and E).

Next, the dependence of the melanosome-aggregating response on the concentration of Ca^{2+} was studied by applying isotonic saline in which the concentration of Ca^{2+} was variously increased. When the extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$) was raised above 30 mM, the melanosomes usually aggregated, and the level of the response

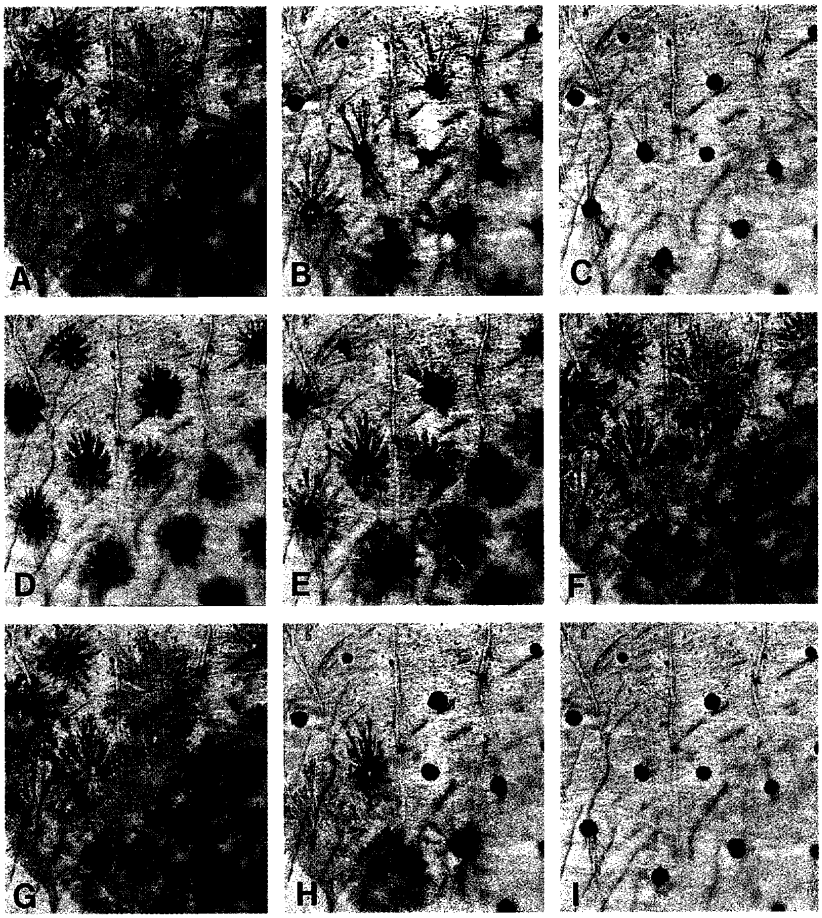

Fig. 1. Serial photomicrographs of the same field, showing the effects of Ca^{2+}-rich saline (Ca^{2+}: 70 mM) on melanophores in an isolated scale from a dark chub, *Zacco temmincki*. (**A**) Equilibrated in physiological solution; melanosomes were completely dispersed in the cells. (**B**, **C**) 3 and 5 min after the application of the Ca^{2+}-rich saline, respectively; melanosomes aggregated into the perikarya. (**D**, **E**) 8.5 and 10 min after the application of the Ca^{2+}-rich saline, respectively; in many melanophores, melanosomes gradually dispersed, while in some cells such as displayed in the upper part the melanosomes again aggregated showing a pulsatile response. (**F**) The skin piece was again equilibrated in physiological saline for 10 min; melanosomes were totally dispersed. (**G**) After pretreatment with 260 nM phentolamine (PA) for 1 min, a Ca^{2+}-rich saline (Ca^{2+}: 70 mM) containing PA at the same strength was applied for 5 min; pigment aggregation was not aroused. (**H**) After washing the scale with physiological saline for 5 min, 50 μM acetylcholine (ACh) was applied for 3 min; melanophores that responded by aggregating their pigment are thought to possess ACh receptors. (**I**) After equilibration in physiological saline for 5 min, 2.5 μM NE was applied for 3 min to induce the maximal aggregation of melanosomes. × 100.

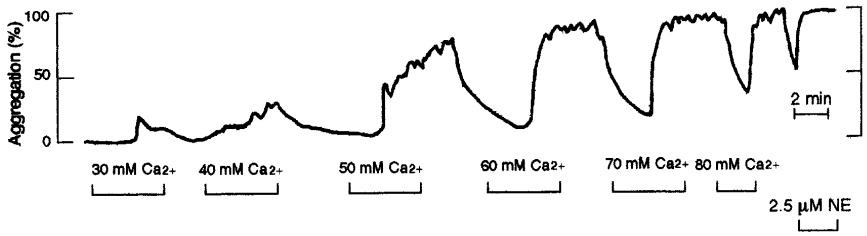

Fig. 2. Typical photoelectric recording of responses to increased $[Ca^{2+}]_o$ concentrations of an individual melanophore in a scale from a dark chub, *Zacco temmincki*. Abscissa; time. The scale is indicated in the right part of the recording. Ordinate: magnitude of melanosome aggregating response. Ca^{2+}-rich saline in which the $[Ca^{2+}]_o$ was raised to 30, 40, 50, 60, and 70 mM were applied in increasing order. Finally, the maximal level of the response was attained by the addition of 2.5 μM NE. Similar explanations for the abscissa and the ordinate apply to the photoelectric recordings in the following Figs.

increased with the concentration of Ca^{2+}, such as shown in Fig. 2. In order to quantify the relationship, NE at a sufficiently strong concentration (2.5 μM) was applied at the end of the experiment, because the NE is known to be very effective in inducing pigment aggregation. Based on the results from a number of such recordings, the concentration-response relationship was drawn, as shown in Fig. 3.

After attaining a certain level of pigment aggregation, the melanophores showed pulsatile responses in the Ca^{2+}-rich saline, such as observed in Fig. 2. Such responses can also be seen in panels B-E of Fig. 1. In this way, the alternating dispersion and aggregation of pigment, or pulsation, took place frequently in the continued Ca^{2+}-enriched environment. In addition, the pulsations seen among many melanophores were mostly synchronized, at least in certain regions of the scale. Panels D, E, and F of Fig. 1 show that melanophores in the right part were in a pigment-dispersed state, while those in the left part were still in the pigment-aggregated state.

Upon returning the perfusing solution to normal physiological saline, melanosomes in the melanophores gradually resumed the fully dispersed state (Fig. 1F). It should also be noted here that the Ca^{2+}-dependent response was not always inducible, and in some cases, the cells in a scale could be refractory to changes in the ionic environment. In some scales, some melanophores were responsive, while others were found to be refractory. At the present time, we remain unable to anticipate which melanophores will be responsive or not, before applying the stimulatory solution.

We then tried to quantify the relation of $[Ca^{2+}]_o$ to the extent of melanosome aggregation induced. Since the melanophores were frequently refractory to increases in the extracellular concentration of divalent cations, it was rather difficult to do so, and generally, about one third of the melanophore population was unresponsive. In this trial therefore, we disregarded data in which melanophores did not respond to the ionic changes. After equilibration in physiological saline, a Ca^{2+}-rich saline was applied for 10 min, in which $[Ca^{2+}]_o$ was variously set from the level in physiological saline up to 85.3 mM (cf. Table 1). The treatment was immediately followed by the application of 2.5 μM NE in normal saline which always induced the maximal level of melanosome aggregation. The level of response attained during the divalent cation-rich saline was expressed as a percentage of the response to NE, and the results are summarized in Fig. 3. The effect can be seen to be concentration-dependent. Discernible aggregation of melanosomes was detected at a concentration of 20 mM, and the maximal level was attained at a concentration of 70 mM.

It should be noted here that even in the same scale, responses to changes in $[Ca^{2+}]_o$ varied considerably among melanophores. Furthermore, in some scales almost all melanophores were responsive, while in others, many of them were refractory.

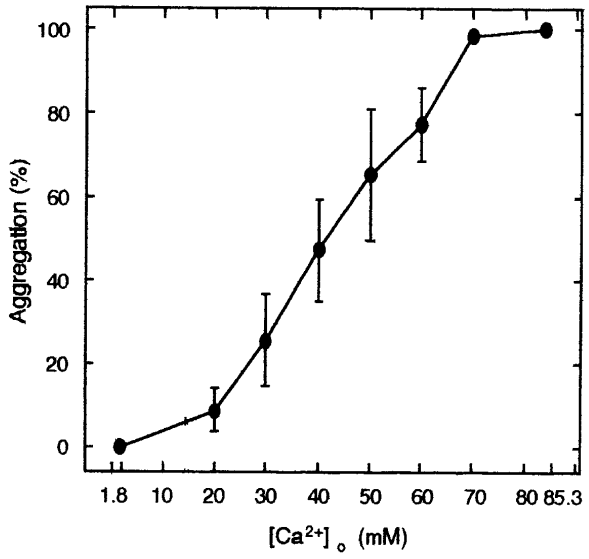

Fig. 3. Relationship between the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and the magnitude of the pigment aggregating response of melanophores from a dark chub. In each measurement contributing to the figure, a Ca^{2+}-rich saline was applied for 10 min, in which the concentration of Ca^{2+} was varied from 1.8 up to 85.4 mM. Then, 2.5 μM NE was applied for 5 min to induce the maximal level of melanosome aggregation. The rate of the response attained during the application of the Ca^{2+}-rich saline is expressed as a percentage of the full pigment aggregation induced by the NE treatment. Abscissa: negative logarithm of the molar concentration of Ca^{2+}. Ordinate: magnitude of response at a percentage of the full aggregation of melanosomes attained during the application of Ca^{2+}-rich saline. Each point represents a mean value with SE (vertical bar). At each concentration, the number of measurements was 7 on the scales from different animals.

Acetylcholine (Ach)-responsive vs. refractory melanophores

It is known that in dark chubs, some melanophores possess ACh receptors of the muscarinic type which mediate the aggregation of melanosomes within them (Hayashi and Fujii, 1993). In that species however, the neurotransmission to melanophores was quite orthodoxly adrenergic, although we remain unaware of the physiological significance of ACh receptors. Of course, there are also many melanophores that lack such cholinoceptors. Before the application of ACh however, we could not predict which cells possessed ACh receptors or not.

With the intent to compare the responsiveness to increases in $[Ca^{2+}]_o$ between these two groups of melanophores, we frequently treated them with ACh. The concentration used was sufficiently strong to elicit pigment aggregation in melanophores, if the cells were endowed with ACh receptors. Notwithstanding the presence of ACh receptors, increases in $[Ca^{2+}]_o$ gave rise to the aggregation of melanosomes (Fig. 1H).

Effects of $[Ca^{2+}]_o$ on denervated melanophores

Denervated melanophores were then tested for their responsiveness to increased Ca^{2+} concentrations. In contrast to normally innervated melanophores, cells deprived of their nervous supply were totally unresponsive to increased

levels of these alkaline metals. A representative result showing this is exhibited in Fig. 4, in which 70 mM Ca^{2+} saline was employed. At the end of the experiment, NE was used to aggregate the pigment. NE was found to be equally effective on denervated melanophores, and further, it induced pigmentary aggregation even in the Ca^{2+}-rich saline (Fig. 4).

Effects of α-adrenolytic agents

Fig. 5 illustrates a photoelectric recording of a melanophore response, in which phentolamine (PA) was employed for its possible inhibitory action on the melanosome-aggregating action of Ca^{2+}. As a control, 70 mM Ca^{2+} saline was applied first. A remarkable aggregation of melanosomes resulted. Next, the scale was again equilibrated in normal physiological saline. Following brief treatment with 260 nM PA, the perfusing medium was changed to 70 mM Ca^{2+} saline that contained PA at the same strength. The response to the increased $[Ca^{2+}]_o$ was completely blocked by PA. After a thorough washing of the drug with normal saline, 70 mM Ca^{2+} saline was again applied to the scale to confirm its responsiveness to the Ca^{2+} elevation. The responsiveness to Ca^{2+} was partially restored. Finally, 2.5 μM NE was administered to elicit the full aggregation of pigment for reference. In contrast, a more specific α_2-adrenergic antagonist, yohimbine (Yoh), α_1-adrenergic antagonist, prazosin, very effectively interfered with these effects. Fig. 5B shows an experiment in which Yoh was employed.

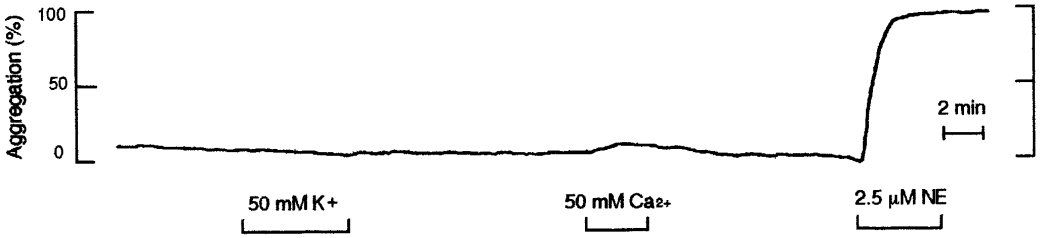

Fig. 4. Typical recording showing the response of a denervated melanophore from a dark chub to an increased level of extracellular Ca^{2+}. Equilibration of the scale in physiological solution was followed by the application of 50 mM K^+ saline for 2 min. The refractoriness of the melanophore indicates that the cell had successfully been denervated. After washing the scale with normal saline, Ca^{2+}-rich saline (Ca^{2+}: 50 mM) was applied. It was also ineffective in inducing melanosome aggregation. As in a normally innervated cell, NE aroused melanosome aggregation.

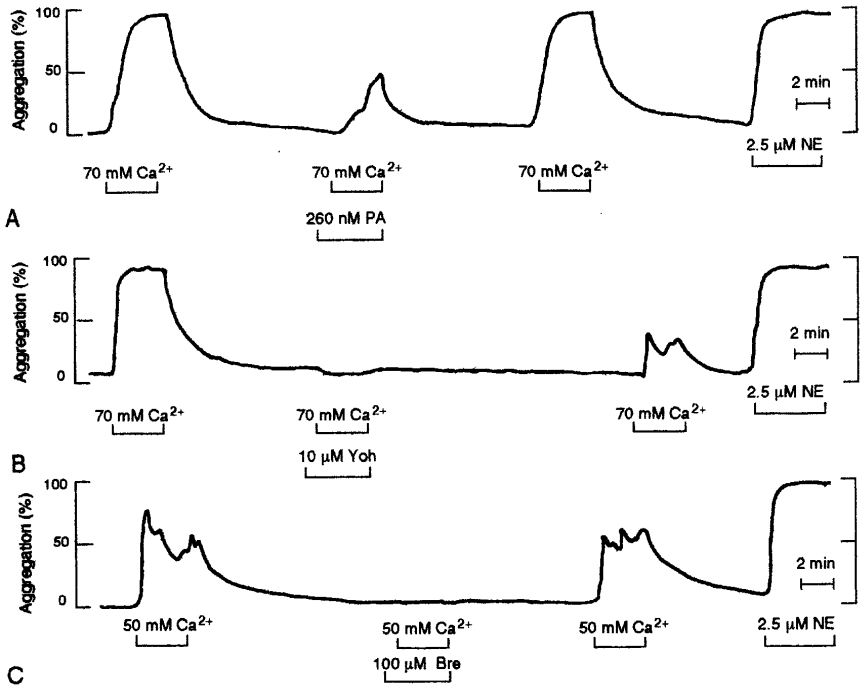

Fig. 5. Typical photoelectric recordings showing the effects of some blocking agents for nervous transmission of the motile responses of an individual melanophore from a dark chub. (**A**) 70 mM Ca^{2+}-saline induced a remarkable aggregation of melanosomes. Phentlamin (PA), a common α-adrenolytic agent, interfered with the action of the Ca^{2+} increase at a comparatively low strength. The effect of PA was reversible, and after washing out the blocker, the normal response to Ca^{2+} was soon restored. (**B**) A more specific α_2-adrenolytic agent, yohimbine (Yoh) was employed. A Ca^{2+}-rich saline (Ca^{2+}: 70 mM) was employed. The blocker interfered with the action of $[Ca^{2+}]_o$ elevation very effectively. (**C**) Bretylium tosylate (Bre), known to block the release of adrenergic transmitter, was used at the concentration of 100 μM. 50 mM Ca^{2+} was used here, and the inhibition was very effective. In this recording, pulsatile responses were remarkable during the action of increased $[Ca^{2+}]_o$.

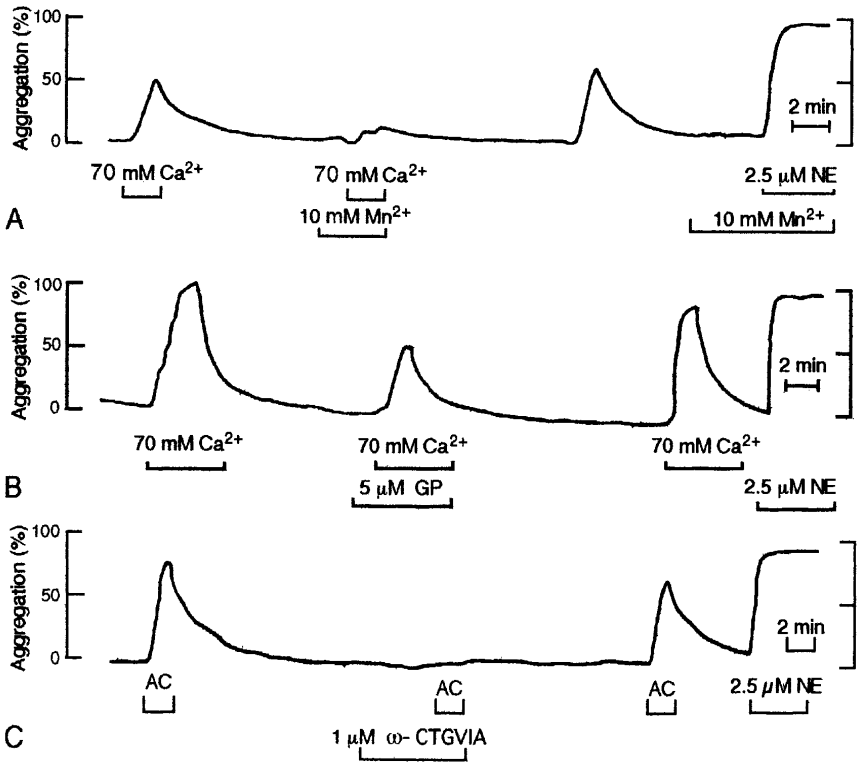

Fig. 6. Typical recordings showing the effect of some Ca^{2+}-channel blockers on motile responses of individual melanophores from a dark chub. (**A**) 10 mM Mn^{2+} was employed; the melanosome aggregating action of 70 mM Ca^{2+} was reversibly blocked by Mn^{2+}. (**B**) 5 μM Gallopamil (GP) was employed; although not so remarkable at this concentration, its inhibition was apparent. The effects were easily recovered following thorough washing out of the drug. Similar results were obtained using verapamil. (**C**) A newly developed very specific voltage-activated Ca^{2+} channel blocker, ω-conotoxin, GVIA (ω-CTGVIA) was used at a concentration of 1 μM. Possibly due to its slow penetration, the onset of the inhibition was much delayed, but the inhibition was remarkable. Electrical stimulation of chromatic fibers was performed by applying alternatiing current (AC).

Effects of a transmitter-release inhibitor

Bretylium (Bre) was tested for its possible inhibition of the $[Ca^{2+}]_o$ -induced release of adrenergic transmitter. As a representative result shows, Bre potently blocked the responses (Fig. 5C). Incidentally, in this particular recording, the pulsatile responses mentioned above can clearly be observed.

Effects of Ca^{2+}-channel blockers

The influences of so-called "Ca^{2+} blockers" were then studied, and typical recordings are displayed in Fig. 6.

First, the effects of Mn^{2+} ions were tested. Following the control response to the increase in $[Ca^{2+}]_o$, the scale was equilibrated in physiological saline to redisperse the melanosomes. Next, physiological saline to which Mn^{2+} ions were added at various strengths was added. This pretreatment was followed by perfusion with a Ca^{2+}-rich saline containing Mn^{2+} at the same concentration. After a few min washing with physiological saline, a sufficiently strong solution of NE was applied to induce the maximal level of melanosome aggregation. It was found that Mn^{2+} at concentrations above 5 mM interfered with the Ca^{2+}-induced aggregation of melanosomes. As a typical result from this type of experiment, Fig. 6A shows a recording in which 10 mM $MnCl_2$ was added to the perfusing medium. In this figure, it can be seen that the responsiveness to Ca^{2+} was partially restored after Mn^{2+} ions were washed out.

Ca^{2+}-channel blockers of the common and general type, verapamil and gallopamil, were then examined. They showed a remarkable ability to block the $[Ca^{2+}]_o$ induced melanosome aggregation. Fig. 6B shows an experiment in which gallopamil was employed.

Finally, a very recently developed agent specific to Ca^{2+}-channels of the N type, ω-conotoxin GVIA, was tested. Presumably due to its larger size, molecules may reach the surface of the presynaptic membrane very gradually, and the onset of effectiveness was unexpectedly delayed. After sufficient equilibration in the toxin-containing saline however, very distinct blockage was seen (Fig. 6C).

DISCUSSION

Instead of the simple NaCl solution, more improved fluids having compositions more closely resembling the body fluids of fish have naturally become popular. When the effects of changing the ionic environment were examined, saline solutions in which Na^+ ions are substituted with other cations have been widely used (e,g., Yamamoto, 1933; Kamada and Kinosita, 1944; Kinosita, 1963; Fujii, 1959; Iwata *et al.*, 1959a). When one of us studied the effects of various cations, including alkaline metal ions (Li^+, K^+, Rb^+, Cs^+) and alkaline-earth metal ions (Sr^{2+}, Ba^{2+}), on melano-

phores of the gluttonous goby, *Chasmichthys gulosus*, salines containing one of those cations replacing Na^+ were employed (Fujii, 1959). As in the first paper by Spaeth (1913), Fujii (1959) also did not report the effects of Ca^{2+} or Mg^{2+}. In fact, we also encountered difficulty in detecting the effects of those cations due to the secretion of large amounts of mucus from epidermal mucous cells during treatment with the ions. Actually, the mucus secretion largely reduced light transmittance through the split fin preparations, thus obstructing the photoelectric recording. In the case of the present materials, the decrease in light transmittance was minimal, allowing us to accurately record the response of melanophores to elevated extracellular concentrations of Ca^{2+} ($[Ca^{2+}]_o$), since the amount of mucus secretion was much less than that secreted from the sticky skin of the goby.

The melanosome-aggregating action of elevated concentrations of K^+ or other cations had been thought to be due to their direct action on the melanophores. Indeed, it was rather natural to think so when one recollects the "potassium contracture" that is ubiquitously observed among striated and smooth muscle tissues. It is now well known that the depolarization of the effector cell membrane due to the effect of high $[K^+]_o$ leads to the muscular contraction via the elevation of intracellular levels of Ca^{2+} ions. Primarily based on observations that denervated melanophores were unresponsive to pigment-aggregating ions, and that the liberation of the sympathetic neurotransmitter was certainly involved in the action on innervated cells, Fujii (1959) first showed that these ions do not act directly on melanophores but rather they act on presynaptic elements of sympathetic postganglionic fibers to release the neurotransmitter. The liberated transmitter then brings about the aggregation of pigment in the melanophore. Working on the crucian carp, *Carassius auratus*, Iwata *et al.* (1959a) soon came to the same conclusion as had Fujii. Adding further evidence for this concept, Fujii (1961) then showed that the effect of K^+ ions could be blocked by treating the skin pieces with an adrenergic blocking agent, dibenamine. The present understanding of the process is that the depolarization due to the heightened $[K^+]_o$ of the presynaptic membrane opens the voltage-dependent Ca^{2+} channels there. The resultant increase in the cytosolic level of Ca^{2+} ions triggers the exocytotic release of the neurotransmitter which finally signals the aggregation of melanosomes in the effector cells (Fujii, 1993). Our goal of the present work was to elucidate whether the mechanism of pigment aggregation in response to an increase in extracellular $[Ca^{2+}]_o$ is identical to that of the action of increasing $[K^+]_o$.

Bearing in mind Nernst's equation, we may naturally expect that an increase of $[K^+]_o$ will result in the profound depolarization of the cell membranes of effector cells as well as of the nervous elements surrounding them. It is now known that various pigment-aggregating or dispersing substances retain their effects even in K^+-rich saline, when melanophores have previously been denervated, or when the release of the transmitter was inhibited (Fujii and Taguchi, 1969; cf. also Fujii, 1993; Fujii and Oshima, 1994). In other words, the motile responses of melanophores seem to be quite independent of the electrical potential across the membrane or of electrical activities there, since under K^+-rich conditions, the membrane should be almost completely depolarized. The conclusion supports the current view that the stimulation of pigment-motor receptors in the cell membrane is transduced via G-proteins, which lead to changes in the intracellular levels of second messengers involved. Ionic channels in the cell membrane, which are directly involved with changes in potential across the membrane, as well as those which are voltage-dependent, seem not to be involved in the process of signal transduction in melanophores (Fujii, 1993). Nevertheless, an increase in $[K^+]_o$ induces the aggregation of pigment in melanophores. As already mentioned above, that action results from the membrane depolarization of presynaptic nervous elements due to the elevated level of $[K^+]_o$ which gives rise to the entry of Ca^{2+} ions through the voltage-dependent Ca^{2+} channels there, which then results in the release of the sympathetic neurotransmitter.

It is well known that changes in the level of intracellular Ca^{2+} ions are critically involved in the motile activities of many types of cells. Chromatophores may not be an exception (cf. Fujii, 1993; Fujii and Oshima, 1994). For example, Luby-Phelps and Porter (1982) showed that the aggregation of pigmentary organelles in erythrophores of the squirrelfish, *Holocentrus ascensionis*, depends on extracellular Ca^{2+} ions, and that their inflow triggers the centripetal displacement of the pigment by increasing the intracellular level of Ca^{2+} ions. Kotz and McNiven (1994), working on erythrophores of the same squirrelfish, showed recently that in addition to low $[Ca^{2+}]_i$, high [cyclic AMP]$_i$ is necessary to induce the dispersion of erythrosomes in the cells.

Working on melanophores of the medaka, *Oryzias latipes*, Negishi and Obika (1985) came to the same conclusion. Using calcium probes, Oshima *et al.* (1988) showed that Ca^{2+} may function to elicit the aggregation of melanosomes in melanophores of the tilapia in culture. Fujii *et al.* (1991) then showed that inositol 1,4,5-trisphosphate acts as a second messenger, in addition to cyclic AMP, which acts via the release of Ca^{2+} from elements of the smooth endoplasmic reticulum to aggregate melanosomes. Having developed a method to simultaneously record motile responses and intracellular changes of Ca^{2+} ions, Toyohara and Fujii (1992) recently succeeded in showing a correlation between the Ca^{2+} increase and the aggregation of melanosomes in tilapia melanophores in culture. Thus, it is quite certain that in many teleost chromatophores the increase in $[Ca^{2+}]_i$ is deeply involved in the aggregation of pigmentary organelles.

In this way, current studies on the functions on Ca^{2+} ions have mostly been concerned with the intracellular involvement of Ca^{2+} ions during pigment aggregation in chromatophores. In contrast, the present study deals with

the effects of Ca^{2+} in the media outside the cells: The elevation of $[Ca^{2+}]_o$ gave rise to the aggregation of pigment in melanophores of all three species of teleosts examined, and denervated melanophores were refractory to that $[Ca^{2+}]_o$ increase. The response was completely blocked by adrenergic α-adrenergic blockers, phentolamine, yohimbine and prazosin, and by bretylium (an inhibitor of the release of the neurotransmitter from adrenergic fibers). It was further shown that Ca^{2+} blockers including Mn^{2+}, verapamil and gallopamil, also blocked the response. These results led us to conclude that the effect of increasing $[Ca^{2+}]_o$ is not on the melanophores themselves, but on the nervous elements that control the aggregation of melanosomes.

The mechanism of generating the resting membrane potential has generally been explained by the so-called "Goldman-Hodgkin-Katz" equation. As is well known, that treatment is based on the unequal distribution of K^+, Na^+ and Cl^- ions across the cell membrane with certain presumed values of permeability coefficients for these ions. In the present study, the experimentally manipulated increase in the extracellular concentration of Ca^{2+} ions is the main issue. Therefore, we wanted to employ an equation that expressed the equilibrium potential in which the changes in $[Ca^{2+}]_o$ are duly incorporated. Fortunately, Yamamoto (1986) has presented a formula in which the equilibration potential (E) can be calculated in terms of the concentrations of Ca^{2+} and Mg^{2+}, in addition to those of the conventional monovalent ions. According to Yamamoto, the equilibrium potential is expressed as:

$$E = \frac{RT}{F} \ln \frac{-b + (b^2 - 4ac)^{1/2}}{2a}$$

where a, b, and c are expressed as:

$$a = [K^+]_i + \frac{P_{Cl}}{P_K}[Cl^-]_o + 4\frac{P_{Mg}}{P_K}[Mg^{2+}]_i + 4\frac{P_{Ca}}{P_K}[Ca^{2+}]_i + \frac{P_{Na}}{P_K}[Na^+]_i,$$

$$b = ([K^+]_i - [K^+]_o) - \frac{P_{Cl}}{P_K}([Cl^-]_i - [Cl^-]_o) + \frac{P_{Na}}{P_K}([Na^+]_i - [Na^+]_o),$$

$$c = -[K^+]_o - \frac{P_{Cl}}{P_K}[Cl^-]_i - \frac{P_{Na}}{P_K}[Na^+]_o - 4\frac{P_{Mg}}{P_K}[Mg^{2+}]_o - 4\frac{P_{Ca}}{P_K}[Ca^{2+}]_o.$$

R, T and F represent the gas constant, the absolute temperature and the Faraday constant, respectively. In our treatment, the temperature was set to be 20°C. Of course, we have no information about the ionic concentrations inside the presynaptic portions of fine sympathetic fibers of fish. However, since our work is concerned with vertebrates, past information on intracellular ionic concentrations as well as permeability coefficients may be assumed to be similar to those existing in postganglionic fibers of the present materials. Therefore, permeability coefficients for Na^+, Ca^{2+}, Mg^{2+} and Cl^- were assigned as 0.04, 0.01, 0.01 and 0.45, respectively, as the relative values when the coefficient for K^+ is taken to be unity. As noted in the Materials and Methods section, an increase in the concentration of Ca^{2+}, namely $CaCl_2$ was compensated by decreasing the same osmolar amount of NaCl to keep the osmolarity of experimental solutions constant. In the present treatment therefore, $[Na^+]_o$ was substituted with "128.0–1.5×$[Ca^{2+}]$" (in mM).

Employing computer graphics, we simulated the effects of changing $[K^+]_o$ on the equilibrium potential across the nervous elements innervating the chromatophores. The trial is not directly concerned with the mechanism of Ca^{2+}-induced pigment aggregation. In addition to the known remarkable pigment aggregating action of K^+ (Spaeth, 1913; Fujii, 1959; cf. also Fujii, 1993), we have conveniently employed a K^+-rich saline in this study to check the integrity of the innervation to the melanophores. The result is graphically presented in Fig. 7A, in which we display cases when P_{Ca} was widely set at 4 steps, since there has been no solid data on P_{Ca} to be used. As in the case where the classical Goldman-Hodgkin-Katz equation is employed, the increase in $[K^+]_o$ effectively depolarizes the membrane potential. When the depolarization reaches the threshold, the voltage-gated Ca^{2+} channels open, resulting in the release of the neurotransmitter from the presynaptic portion. In any case, the simulation definitely shows that an increase in $[K^+]_o$ is effectively depolarizing. The theoretical interpretation of the K^+-induced pigment aggregation may thus be provided for our earlier conclusion about the mechanism of K^+ action (Fujii, 1959). Judging from the curves in Fig. 7A furthermore, we can also learn that the increase in P_{Ca} is not such an important factor in the K^+-dependent depolarization.

Using Yamamoto's equation, we then tried to simulate the effects of increasing $[Ca^{2+}]_o$ as exhibited in Fig. 7B. In this panel, we assumed 3 values of P_{Ca}. When the P_{Ca} is very low, the effects of increasing $[Ca^{2+}]_o$ are naturally small. When it is 0.01, even a slight hyperpolarization is observable. Apparently, this was due to a decrease in $[Na^+]_o$ in the solution used to bathe the cells. Upon increasing the P_{Ca}, an increase in $[Ca^{2+}]_o$ tended to depolarize the membrane more effectively. When the P_{Ca} is assumed to be 1.0, the depolarization due to the increase in $[Ca^{2+}]_o$ becomes remarkable, and is supposed to open the voltage-dependent Ca^{2+} channels there with higher probability.

By opening those Ca^{2+} channels, the P_{Ca} naturally increases, which induces the upward transition of the curves in Fig. 7B. Such a process can be more clearly seen in Fig. 7C, where the increase in P_{Ca} is put on the abscissa. The figure clearly shows that the increase in P_{Ca} accelerates depolarization of the membrane, and that, when the $[Ca^{2+}]_o$ is above 30 mM or so, the membrane depolarizes very quickly in response to an increase in the Ca^{2+} permeable coefficient. Such a result explains quite easily the observed concentration-dependent pigment-aggregating action of $[Ca^{2+}]_o$ (Fig. 3). As a result of such a self-regenerative process, the intracellular concentration of Ca^{2+} may easily reach the threshold level to allow the release of the neurotransmitter.

Another point of interest in the action of the $[Ca^{2+}]_o$ increase is that the effects vary quite a lot among the melanophores (Fig. 1). Such a differential responsiveness may be due to differences in the thickness (density) of innerva-

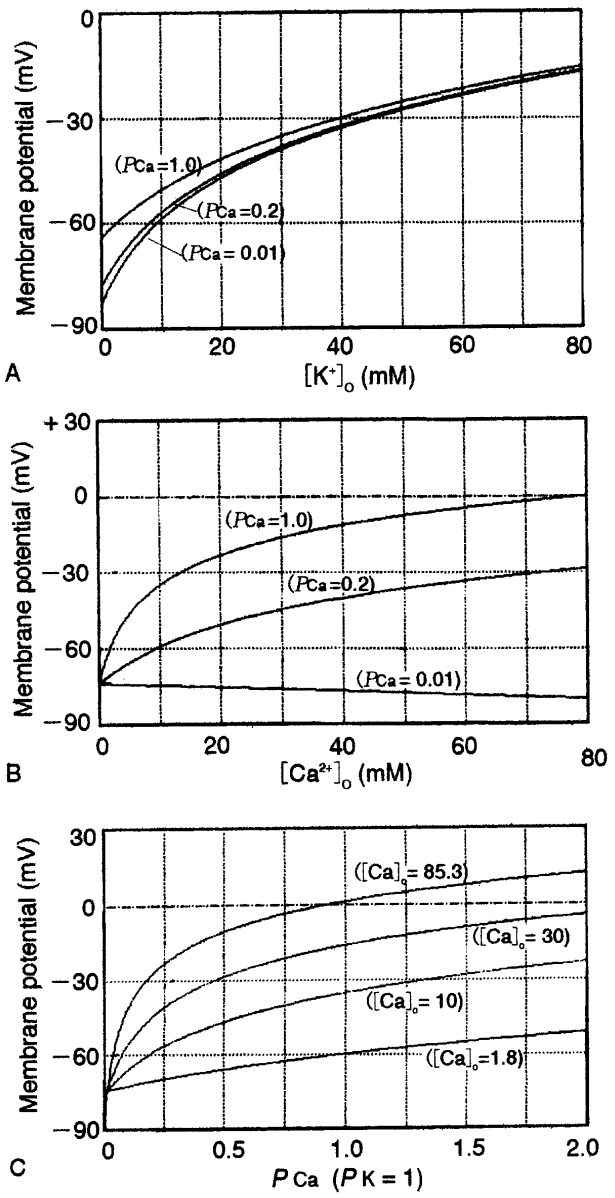

Fig. 7. Simulated curves by computer graphics for analyzing possible influences of $[Ca^{2+}]_o$ on the equilibrium potential across nervous elements innervating the chromatophores. (**A**) Effects of changing $[K^+]_o$ for reference on the equilibrium potential across the nervous elements innervating the chromatophores. *P*Ca was widely set at 3 steps, i.e. 0.01, 0.2 and 1.0. The increase in $[K^+]_o$ effectively depolarizes the membrane potential. (**B**) Effects on the membrane potential of increasing *P*Ca. Three values of *P*Ca, namely, 0.01, 0.2 and 1.0, were used here. Upon increasing the *P*Ca, the depolarization due to $[Ca^{2+}]_o$ depolarizes the membrane more effectively, resulting in an opening of the voltage-dependent Ca^{2+} channels there. (**C**) Here, the increase in *P*Ca is put on the abscissa from 0 to 2.0. It is clearly shown that an increase in *P*Ca accelerates depolarization of the membrane.

tion to individual melanophores, since the sensitivity to norepinephrine is quite even among melanophores. When a melanophore is richly supplied with nerves, the amount of neurotransmitter released around the cell should be high, which would result in a large response of melanosome aggregation. When a cell is poorly innervated or lacks a nervous supply, the response would be smaller or no response would be observed.

In catfish, which belong to the family Siluridae, peripheral neurotransmission has been disclosed to be strangely cholinergic, although the innervation is sympathetic postganglionic (Fujii and Miyashita, 1976, 1982; Fujii, 1993). Using the Asian catfish *Silurus asotus* (Fujii *et al.*, 1982) or the translucent glass catfish *Kryptopterus bicirrhis* (Kasukawa and Fujii, 1984), we were also able to detect the melanosome-aggregating action of K^+, and have concluded that the mode of action is quite similar to that in the usual adrenergically innervated melanophores. Working on the two cyprinid species furthermore, Hayashi and Fujii (1993) recently showed that some melanophores are endowed with cholinoceptors. Both species belong to the same genus *Zacco*, and one of them (*Z. temmincki*) is the species on which the greater part of the present work has been performed. Taking this into consideration, we performed experiments on melanophores of this species, but could not detect any difference in the responsiveness between melanophores with or without cholinoceptors. As for the effect of increasing $[Ca^{2+}]_o$ therefore, the presence of cholinoceptors in the effector membrane may have no relevance to the pigment-aggregating action of extracellular Ca^{2+} ions.

As already touched upon, changes in the $[Ca^{2+}]_i$ are generally concerned with the motile responses of chromatophores. The problem is whether the Ca^{2+} ions are derived from the extracellular space or from an intracellular Ca^{2+}-storage compartment, such as the smooth endoplasmic reticulum. In squirrelfish erythrophores, dependence on $[Ca^{2+}]$ has been clearly shown (Luby-Phelps and Porter, 1982). Using squirrelfish from the Japanese coastal waters, we were able to confirm that phenomenon (Fujii, 2000b). In contrast, results on melanophores, leucophores, and motile iridophores were completely different (cf. Fujii, 1993, 2000a). For example, our earlier results on the gluttonous goby, *Chasmichthys gulosus*, clearly indicated that the motile activities of melanophores are independent of the presence of extracellular Ca^{2+} (Fujii and Taguchi, 1969). In these chromatophores therefore, changes in the intracellular level of Ca^{2+} come at the expense of intracellularly deposited Ca^{2+} ions independently of the extracellular concentration of the ions. The present analyses on the involvement of Ca^{2+} ions on the membrane potential based on the formula presented by Yamamoto (1986) indicates that the cell membranes either of the nervous elements or of the melanophores may be largely depolarized in Ca^{2+}-rich saline. Such phenomena are consistent with the current understanding that the motile responses of melanophores are mostly mediated by G-protein linked membrane receptors which can be functionally independent of the membrane potential (cf. Fujii, 1993, 2000a). In addition to Ca^{2+} ions, adenosine 3';5'-cyclic monophosphate (cAMP) and inositol 1,4,5 trisphosphate (IP_3) are known to be the principal second messengers in these processes (Fujii, 1993, 2000).

In conclusion, the mechanism of the melanosome-aggregating action of increasing $[Ca^{2+}]_o$ in teleosts is quite analogous to that of $[K^+]_o$, and can be diagrammed as

shown in panel A of Fig. 8. We consider that the mechanism may be generally applicable to melanophores, leucophores, and motile iridophores, and might also be functional in many brightly-colored chromatophores as well. As for squirrelfish erythrophores (Luby-Phelps and Porter, 1982; Fujii, 2000b), medaka xanthophores (Oshima *et al.*, 1998), and some others, the diagram shown in Figure 8B may be used, which was drawn based on the currently available information on these bright-colored chromatophores.

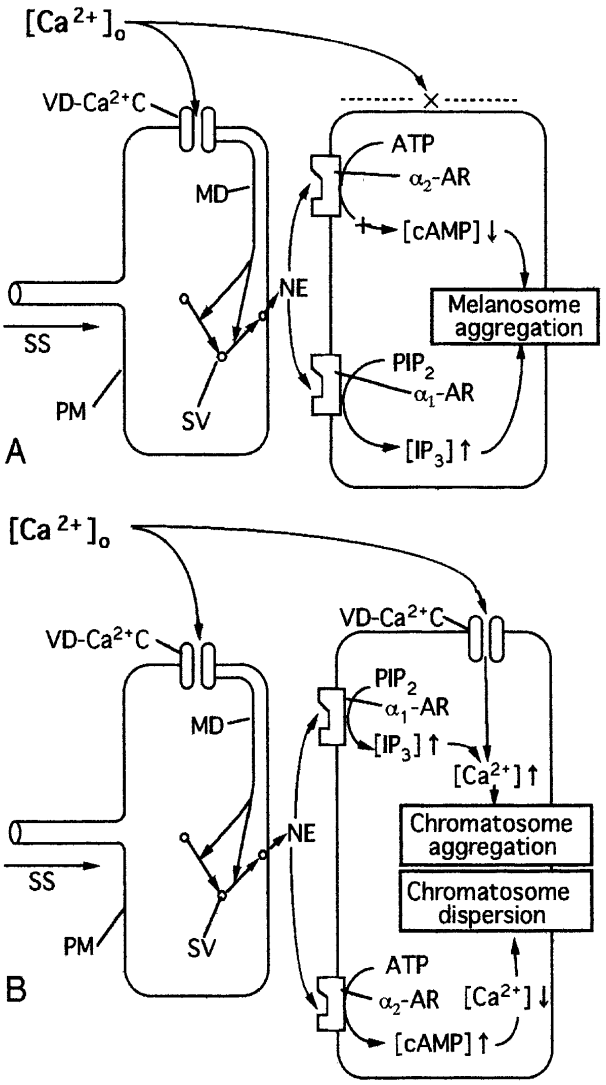

Fig. 8. Diagrams summarizing the conclusions of this study about mechanism of melanosome-aggregating action of increasing $[Ca^{2+}]_o$ in teleosts. (**A**) General mechanism of the action of a $[Ca^{2+}]_o$ elevation on most teleostean chromatophores, including melanophores, leucophores, and motile iridophores, and possibly many brightly-colored chromatophores. (**B**) Mechanism of action of the $[Ca^{2+}]_o$ elevation in squirrelfish erythrophores, medaka xanthophores and possibly in some brightly-colored chromatophores in fishes belonging to teleosts, based on currently available information. Although much more specific than that shown in **A**, it should be taken in consideration when we discuss relevant issues thereafter. α_1-AR: α_1-adrenoceptor, α_2-AR: α_2-adrenoceptor, cAMP: cyclic adenosine-3',5'-monophosphate, IP_3: inositol 1,4,5-trisphosphate, MD: membrane depolarization, PIP_2: phosphatidyl inositol 4,5-bisphosphate, PM: presynaptic membrane, SS: sympathetic signal, SV: synaptic vesicle, VD-Ca^{2+}C: voltage-dependent Ca^{2+} channel.

ACKNOWLEDGMENTS

We thank Professor M. Yoshino, Department of Biology, Tokyo Gakugei University, for valuable advice, and Professor N. Oshima and Dr. M. Sugimoto for their interest and helpful discussions. We would also like to acknowledge the Director, Dr. T. Miyamori, and the staff of the Pariative Care Unit, Ida Hospital, for generously allowing us to perform final parts of the work including preparation of the manuscript. Finally, special thanks are due to Mrs. Y. Fujii for her continuing support, and to Mr. T. Harada and Mrs. S. Harada for their unfailing assistance.

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(Received March 5, 2002 / Accepted May 22, 2002)