Effects of Grayanotoxin on the Fish Melanophore System

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Abstract
α-Dihydrograyanotoxin II (α-2H-GTX II) induced melanosome aggregation in the innervated melanophores of a teleost, Zacco temmincki, but not in the denervated ones. The effect of the toxin was inhibited by tetrodotoxin (TTX). It was concluded that α-2H-GTX II acted on the nerve to open Na-channels of the membrane, producing transmitter release from varicosities of the chromatic fiber. Since α-2H-GTX II and TTX did not affect responsiveness of melanophores to noradrenaline, it was argued that Na+ inflow through the membrane did not participate directly in triggering pigment movements.

Introduction
It has been widely recognized that melanophores in many teleost fishes receive the supply of adrenergic neurons (Reed and Finnin, 1972; Fujii and Novales, 1972; Bagnara and Hadley, 1973; Fernando and Grove, 1974; Fuji and Miyashita, 1975). Isolated skin preparations, thus, are a system composed of melanophores and their nerves. Many investigations have been concentrated on the melanophores as an effector, and a little is known about properties of the nerves (Fujii and Novales, 1972). As it is easy to obtain denervated preparations in teleost melanophores, the skin preparations are suitable for investigating action points of various stimuli.

It has been known that grayanotoxins, extracted from the leaves of the family Ericaceae, open specifically the Na-channel on the membranes and depolarize those of Na-dependent excitable cells (Starkus and Narahashi, 1978; Seyama, 1978). In order to clear some properties of the fish chromatic nerve, and to know a relationship between Na-channels and movements of melanosomes, effects of α-2H-GTX II, one of the most potent grayanotoxins, on the melanophores and their nerves were investigated.

Materials and Methods
Scale melanophores of a cyprinid fish, Zacco temmincki, were used. Scales were isolated from an area within the dark band of the fish. Denervated melanophores were obtained from animals intraperitoneally injected 6-hydroxydopamine (80 µg/g)
The experimental methods and procedures were essentially the same as those described in a previous paper (Iga and Matsuno, 1980). AC stimulation (60 Hz) was performed by a pair of Pt plate placed at a distance of 3 mm. A physiological solution had a following composition; 128 mM NaCl, 2.6 mM KCl, 1.8 mM CaCl₂, 5 mM Tris-HCl buffer (pH 7.2). The drugs used were noradrenaline hydrochloride (Sigma Chemical Co.), tetrodotoxin (Sankyo Co., LTD) and α-dihydrograyanotoxin II (which was kindly provided by Dr. Seyama).

All experiments were performed at a room temperature between 20–25°C.

Results

1. Effect of α–2H–GTX II on the innervated melanophores

The melanophores in the isolated scales maintain a dispersed state in physiological solution. The scales were put in test solutions containing α–2H–GTX II in various concentrations. This toxin caused a pigment aggregation in the innervated melanophores after a certain time of the application; the time required for beginning of melanosome aggregation was dependent on the concentrations of α–2H–GTX II. The time was 1.9 ± 0.5 min, 4.7 ± 2.6 min, 9.2 ± 4.0 min and 16.5 ± 10.0 min at concentrations of 10⁻⁴M, 5 × 10⁻⁵M, 3 × 10⁻⁵M and 10⁻⁵M, respectively. During the aggregating process, small pulsating movements of the melanosomes were observed, especially in a low concentration of the toxin. Figure 1 illustrates a typical recording in each concentration of α–2H–GTX II.

![Melanosome aggregation in the innervated melanophores at various concentrations of α–2H–GTX II.](image-url)
The pigment aggregation induced by the toxin lasted for a fairly long time even after the solution was exchanged to the physiological solution.

2. Effect of α-2H-GTX II on the denervated melanophores

In order to search the action point(s) of the toxin which induced the melanosome aggregation in the innervated melanophores, the same experiment was done on the denervated ones. No pigment aggregation was induced on the denervated ones even for 10 min treatment with the toxin of 10^{-4}M, which induced melanosome aggregation of the innervated melanophores within 2 min (Fig. 2). The pretreatment did not significantly affect the responsiveness of the melanophores to noradrenaline (Fig. 2 and 3). A pretreatment with 10^{-6}M TTX for 10 min also did not significantly change the sensitivity of the melanophores to noradrenaline (Fig. 3).

![Fig. 2. Effect of α-2H-GTX II on the denervated melanophores.](image)

![Fig. 3. Effects of α-2H-GTX II or TTX-pretreatment on the melanosome-aggregating response of the denervated melanophores to noradrenaline.](image)
3. Effects of TTX on the α-2H-GTX II action

From the experiments of section 2, it is possible to conclude that α-2H-GTX II acts on the pigment-aggregating nerves to promote the release of the transmitter which causes melanosome aggregation. As it is known that grayanotoxins generate excitation through the opening of Na-channels on some excitable membranes, as described in the introduction, a relation between this toxin and TTX, a closer of the Na-channel, was investigated.

In this experiment, the innervated melanophores were exclusively employed. The response of the melanophores to AC stimulation was inhibited by the treatment with 10⁻⁶M TTX, while that of the melanophores to 5 K (physiological solution: 1/7.5 M KCl = 1:1) was not affected (Fig. 4a). These results show that the site affected by KCl and AC is different; K ions specifically acts on the nerve terminals (varicosities) and AC on the nerve fibers (Fujii and Novales, 1968; Iga, 1982). The effect of TTX was continued at least for 30 min (Fig. 4b).

Then, it was examined how TTX could affect the aggregation response induced by α-2H-GTX II. When the melanophores were incubated in a test solution containing both 10⁻⁶M TTX and 10⁻⁴M α-2H-GTX II, the action of α-2H-GTX II was never appeared (Fig. 5a). The action of α-2H-GTX II was completely inhibited also by the pretreatment with 10⁻⁶M TTX (Fig. 5b). Figure 5 also shows that the action of TTX could not be affected by the coexistence of α-2H-GTX II (Fig. 5a) or its posttreatment (Fig. 5b). 10⁻³M TTX produced melanosome dispersion of the melanophores which had kept aggregation of their melanosomes under the effect of GTX (Fig. 6).
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Fig. 5. Inhibition of the melanosome aggregating action of α-2H-GTX II by TTX. (a) coexistent application of both toxins, (b) pretreatment with TTX.

Fig. 6. Antagonistic effect of TTX on the melanosome aggregating action of α-2H-GTX II.

Discussion

It is widely known that the melanophore-controlling nerve in many teleost fishes is of an adrenergic nature (Reed and Finnin, 1972; Fujii and Novales, 1972; Bagnara and Hadley, 1973; Fernando and Grove, 1974; Fujii and Miyashita, 1975). α-2H-GTX II caused pigment aggregation in the innervated melanophores, but not in the denervated ones. Furthermore, this toxin did not affect the sensitivity of the melanophores to noradrenaline. The same was in the treatment with TTX. This indicates that α-2H-GTX II can not directly affect the melanosome movement of the cell, but acts on the nerve to release the transmitter from the varicosities.

The melanosome aggregation induced by α-2H-GTX II was completely suppressed by TTX. This result, as well as in some Na-dependent excitable membranes (Starkus and Narahashi, 1978; Seyama, 1978), indicates that α-2H-GTX II acts to open Na-
channels on the membrane of the chromatic nerve, and that a resultant Na\(^+\) influx induces the release of transmitter from the varicosities, while an inflow of Na\(^+\) through the melanophore membrane can not directly take part in the melanosome movement.

The pigment aggregation induced by \(\alpha\)-2H–GTX II continued for a fairly long time, even after the toxin had been washed out. This may indicate that the action of \(\alpha\)-2H–GTX II is irreversible. TTX, however, introduced the GTX-induced aggregation to the dispersion. Considering that TTX could not cause melanosome dispersion of the melanophores which had been aggregated by noradrenaline, it may be natural to consider that the continuous aggregation of melanosomes is maintained by a continuous release of transmitter from the nerve varicosities by the action of \(\alpha\)-2H–GTX II, but not by an enormous, temporal release of the transmitter.

It has been known that the release of transmitter from nerve terminals is dependent on the inflow of extracellular Ca\(^++\) (Katz and Miledi, 1967; Llinas et al., 1972; Miledi, 1973; Blaustein, 1975). As we have confirmed that an extracellular Ca\(^++\) is also necessary for the transmitter release in the chromatic nerve of the teleost Zacco temmincki (Iga and Takabatake, unpublished), it is suggested that the lasting liberation of the transmitter requires a continuous supply of Ca\(^++\). Experiments from this viewpoint are under way in our laboratory.

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**References**


