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The Effect of Ouabain on Melanophore Movements in a Freshwater Teleost, *Oryzias latipes*

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Abstract

Ouabain, a sodium- and potassium-dependent ATPase inhibitor, induced irreversible pigment aggregation in the innervated melanophores of *Oryzias latipes*, but in the denervated ones it failed to cause pigment aggregation or pigment dispersion, suggesting that this drug acts on the presynaptic nervous elements to promote spontaneous release of the transmitter which produces pigment aggregation within melanophores. Since ouabain did not affect responsiveness of the melanophores to directly acting substances, it was concluded that a Na–K ATPase did not participate directly in pigment movements. The effect of ouabain on the nervous elements was antagonized by excess potassium ions in the external medium.

Several hypotheses have been proposed to explain pigment movements within melanophores in teleost fishes (cf. Fujii and Novales, 1969; Bagnara and Hadley, 1973). Since microtubules were observed with an ordered array in melanophores in a variety of teleost fishes, their participations on the pigment migration have been actively discussed (Bikle et al., 1966; Green, 1968; Wikswo and Novales, 1972; Schliwa and Bereiter-Hahn, 1973a, b, 1975; Murphy and Tilney, 1974; Schliwa, 1978; Schliwa et al., 1978).

Microtubules in various kinds of cells are composed of proteins containing sulfhydryl groups (Kawamura, 1960; Sakai, Kuriyama and Kimura, 1973; Stephens and Edds, 1976; Synder and McIntosh, 1976). In *Oryzias* melanophores, sulfhydryl inhibitors, Mersalyl and NEM, blocked effectively pigment aggregation within the melanophores, suggesting that SH-proteins play some important roles on pigment migration (Iga, 1975). However, the sites affected by these drugs have remained unsolved whether they are the structural entities like microtubules, SH enzymes *e.g.* ATPase, or any other functional sites in the membrane.

At the present experiments, the effect of ouabain, a specific inhibitor of Na–K ATPase, on melanophore movements was investigated and some participation of a Na-pump on the melanophore activity was also discussed.

Materials and Methods

Melanophores in an isolated scale of a freshwater teleost, *Oryzias latipes*, were utilized as the experimental material. Both innervated and denervated melanophores were used. The denervated preparations were obtained by the same method described previously (Iga, 1968, 1975a). A scale removed from the dorso-lateral trunk of the fish was held, epidermal side down, on the under surface of a cover glass, which was mounted on a perfusion chamber which was filled with physiological saline solution of the following composition: 128 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$ (pH $=7.2$ by $NaHCO_3$). In the perfusion chamber the preparation was irrigated continuously with saline or experimental solutions.

The KCl solution was isotonic with the physiological saline (pH $=7.2$ by $KHCO_3$). The following drugs were used: *l*-adrenaline hydrochloride (Adrenaline-injection fluid, Sankyo, Tokyo), atropine sulfate (Kanto, Tokyo) and ouabain (Merck, Dermstadt). These drugs were disolved in the physiological saline and applied to the scale preparation as external solutions.

The method of recording melanophore response was fundamentally the same as that described by Iga (1975b). Changes in light transmission due to melanophore responses exposed to various solutions were converted into those in photoelectric current by a Cds photoconductive cell, which was attached behind one eyepiece of a microscope with a trinocular assembly, and were recorded on a paper chart recorder (Yokogawa, 3046), where the upward deflection of the trace was set to indicate the increase in transmittance, that is, the melanosome aggregation, while the downward deflection represented melanosome dispersal. Simultaneous visual observations were made with the other eyepiece. In addition to a blue filter (LB–45), a yellow filter (Y–48) was inserted between the light source and the condenser lens to eliminate light variations due to the concurrent xanthophore responses. By putting a circular diaphragm at the plane of the real image inside the eyepiece, the area of the scale through which light was transmitted was restricted to about 130 μ diameter. Thus, the response of a single melanophore could be measured.

All experiments were performed at room temperature between 20–23°C.

Results

Effect of ouabain on the innervated melanophores

Melanophores immersed in physiological saline disperse from an aggregated state and maintain pigment dispersal. In innervated melanophores, however, the pigmentary state is interrupted transiently with spontaneous pigment aggregation within the melanophores in certain hours after isolation of the scale, reaching a punctate state,

and then regains the initial dispersion state (Iwata et al., 1959; Iga, 1975b). In the present observation, the spontaneous pigment aggregation in the melanophores began around 2 hrs after the scale isolation.

Fifteen min after the isolation, a complete pigment aggregation within the melanophores by KCl was confirmed (type 1 melanophore, Iga, 1975a), and then the scale was perfused with physiological saline. Thus, 30 min after the scale isolation, the scales were immersed in test solutions containing ouabain in various concentrations (10^{-3}–10^{-7} M). Ouabain affected to induce pigment aggregation within the melanophores after a certain time: the time required for beginning of melanosome aggregation was dependent on the concentrations of ouabain. In 10^{-3} M ouabain, the melanosome aggregation began within 3 min. In 10^{-6} M, the melanosome aggregation commenced around 40 min after treatment with ouabain. At lower concentration (10^{-7} M), ouabain had little or no effect on hastening the pigment aggregating phase. The aggregation was accompanied with small pulsating movements of the melanosomes until it attained a full state. Figure 1 illustrated a typical recording in each concentration of ouabain and also these results were summarized in Table 1 where the time in control was indicated as that passed 30 min after the scale isolation.

The pigment aggregation induced by ouabain lasted for a fairly long time even after the ouabain solution was exchanged to the saline alone. If the KCl solution was applied to the melanophores which had maintained melanosome aggregation in physiological saline under the after effect of ouabain, the melanophores responded with rapid pigment dispersion (Fig. 2).

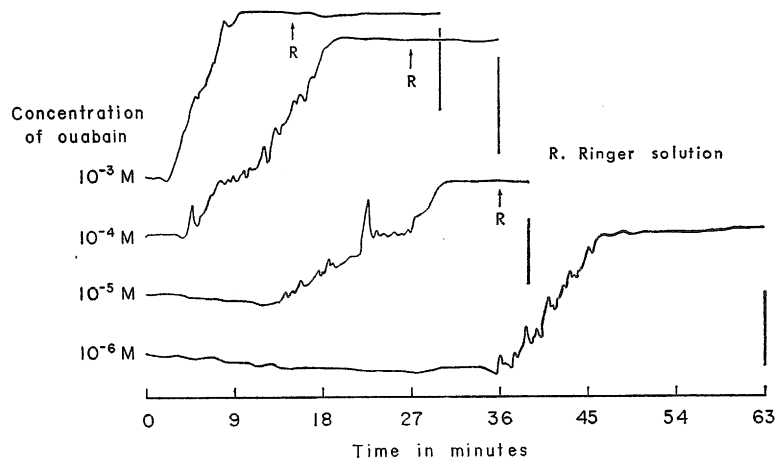


Fig. 1. Melanosome aggregation in the innervated melanophores during continuous immersion in various concentrations of ouabain. Vertical bars at the right side of each response curve indicate the magnitude of 50% aggregation of melanosomes in the corresponding curve, respectively.

Table 1. Comparison of the time required for the beginning of melanosome aggregation in the innervated melanophores to various concentrations of ouabain. Number in parenthesis represents number of experiments, respectively.

Concentration of ouabain	Time required for the beginning of melanosome aggregation	
10^{-3} M	2.6±0.20 min.	(8)
10^{-4}	3.3±1.55	(8)
10^{-5}	10.3±2.64	(9)
10^{-6}	36.9±4.22	(8)
0 (Control)	79.2±3.17	(8)

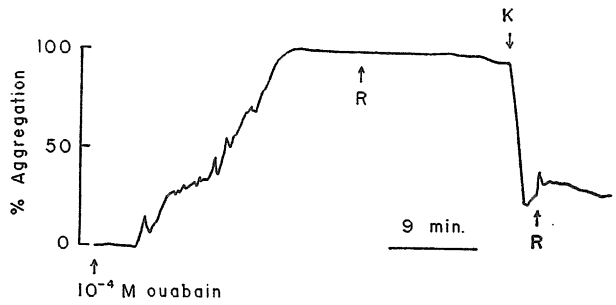
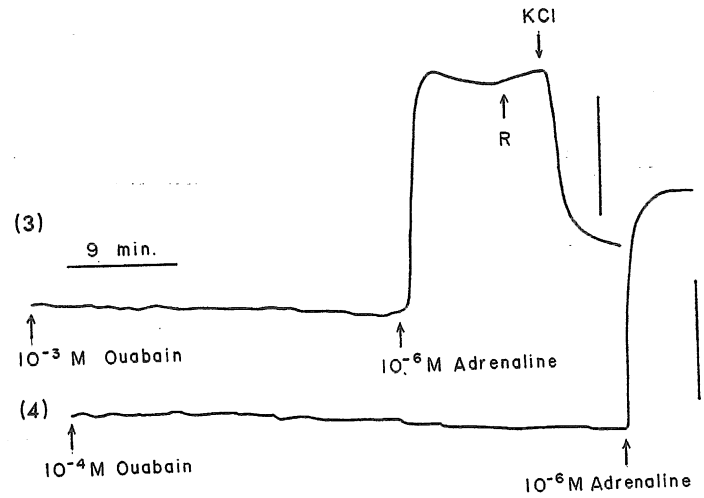


Fig. 2. Irreversible functional loss of the pigment aggregating nerve by ouabain treatment.

Effect of ouabain on the denervated melanophores

Success of denervation is confirmed by lack of a pigment aggregating response to KCl and by supersensitization to adrenaline (Iga, 1968, 1975b). In the denervated melanophores no pigment aggregation nor pigment dispersion was induced during the incubation with ouabain (10^{-3}–10^{-6} M). Furthermore, the preincubation with ouabain did not affect the responsiveness of the melanophores to adrenaline; 10^{-6} M adrenaline induced rapid pigment aggregation with the maximal level (Figs. 3 and 4). Even after incubation in 10^{-4} M ouabain for 5 hrs, the melanophores showed to still normally be able to respond with pigment aggregation to adrenaline.

Ouabain had also no effect on the pigment dispersing response to atropine or KCl, which acted directly on the melanophores (Figs. 3 and 5).



Figs. 3 and 4. State of denervated melanophores during continuous immersion in ouabain and the response of the melanophores to adrenaline. Vertical bars at the right side of each response curve indicate the magnitude of 50% aggregation of melanosomes in the corresponding curve, respectively.

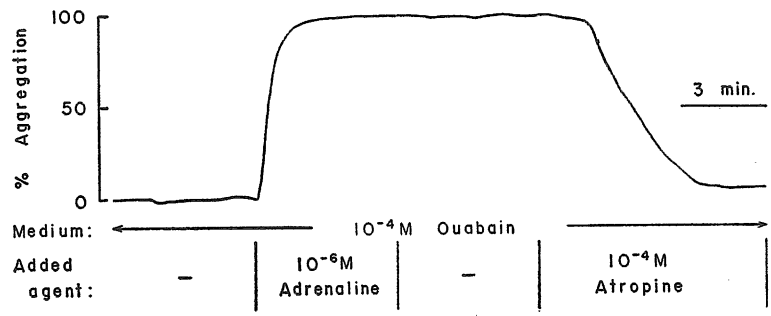


Fig. 5. Effect of ouabain on melanosome movements in the denervated melanophores. The scale was incubated for 15 min in a solution of 10^{-4} M ouabain prior to adrenaline application.

The effect of high K solution on the action of ouabain

As mentioned above, pigment aggregation induced by ouabain was kept in a punctate state for a fairly long time even after the test solution was replaced with physiological saline (Fig. 6a). If 5×10^{-4} M ouabain-5K solution, a mixture of 10^{-3} M ouabain and the KCl solution in equivalent volume, was applied to the innervated melanophores in stead of 5×10^{-4} M ouabain solution, the melanophores responded with rapid pigment aggregation in exactly the same manner of response to the KCl solution alone and maintained their punctate state. On return to physiological saline,

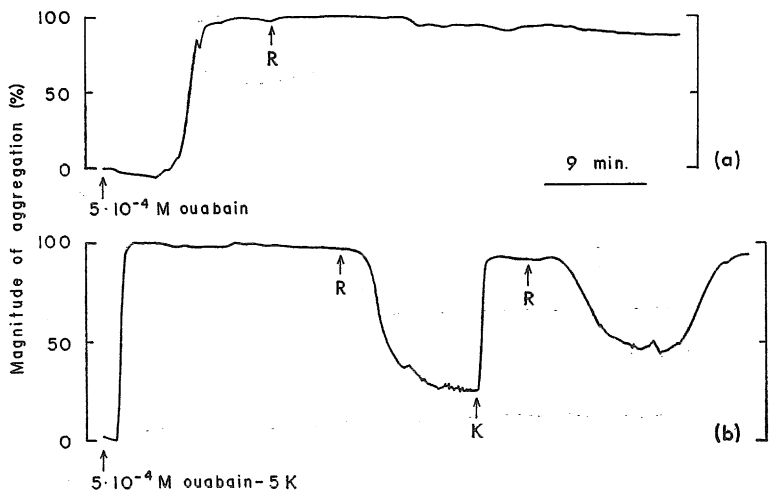


Fig. 6. Inhibition of the action of ouabain by high K solution in the innervated melanophores (b). (a), control.

in this case, the melanosomes in the melanophores dispersed with a normal process and the dispersed melanophores responded to subsequent application of KCl with rapid pigment aggregation. Replacement of the KCl solution to saline caused again pigment dispersion within the melanophores (Fig. 6b). Thus, the pigment aggregation action of ouabain on the innervated melanophores was inhibited in the 5K solution.

Discussion

It is well known that melanophores in many teleost fishes are under the control of the autonomic nervous system (Parker, 1948; Fujii and Novales, 1972; Bagnara and Hadley, 1973). Since the denervated melanophores are obtained with ease and certainty in the scales (Iga, 1968, 1975b) or in the tail fins (Fujii, 1959) of the fishes, fish melanophores are elegant preparations for physiological and pharmacological studies on action mechanisms of various stimuli to effectors with innervation.

At the present experiments, the result that ouabain caused pigment aggregation only in innervated melanophores suggested that ouabain affected indirectly on the melanophores through an intermediation of the presynaptic nervous elements. The melanophores treated with ouabain responded with rapid pigment dispersion, instead of pigment aggregation, to KCl. This dispersion induced by KCl implied that the melanophores were functionally under denervated state (Iga, 1976, 1977). Thus, ouabain brought about irreversibly the functional loss of the pigment aggregating nerve. Since it has been reported that ouabain facilitates the spontaneous and stimulated release of catecholamines from the neuronal and extraneuronal sites in a

variety of adrenergic organs in vitro (Banks, 1967; Garcia and Kirpekar, 1973a, b; Ozawa and Katsuragi, 1974; Katsuragi and Suzuki, 1976; Duncan, 1976), it is possible to concluded that also in the present experiments ouabain acts on the pigment aggregating nerve terminals to promote spontaneous release of the transmitter which causes pigment aggregation. The time required for inducing pigment aggregation progressively shortened as the concentration of ouabain increased in the range of 10^{-6} to 10^{-3} M. Thus, the spontaneous pigment aggregation in the melanophores in isolated preparations during continuous immersion in physiological saline might be caused by an increase of spontaneous leakage of the transmitter substances from the presynaptic terminals, whose leakage was promoted by a decrease in the activity of Na pump brought through a prolonged immersion of isolated preparations in physiological saline. Watanabe (1960) observed that KCN, a respiratory inhibitor, shortened the time course of the initial dispersion phase of the melanophores in the isolated scales of the crucian carp, *Carassius auratus*.

The effect of ouabain on the innervated melanophores was inhibited by a high K solution. The antagonism of potassium ions on ouabain effect is well recognized in both isolated enzyme preparations and intact cells (Glynn, 1964; Schwartz et al., 1975; Shaver and Stirling, 1978).

Ouabain could not induce pigment aggregation nor pigment dispersion in denervated melanophores and the long-term (5 hrs) treatment with it also did not affect the responsiveness of the melanophores to both pigment-aggregating and pigment-dispersing substances. Therefore, it can be rather safely concluded that ouabain has essentially no effect on melanophores through interference with pigment movements or with their responsiveness to directly acting substances. These results indicate that the membrane Na–K ATPase does not directly take part in pigment movements.

Egner (1971) shortly mentioned that ouabain induced pigment aggregation with pulsation in the melanophores of the angelfish, *Pterophyllum scalare*. On the contrary, ouabain and strophantidin promoted reversibly an evident blocking of pigment migratory process of the melanophores in *Fundulus heteroclitus* (Junqueira and Porter, 1974). On the basis of this result, they concluded that a sodium- and potassium-dependent ATPase participated somehow in pigment migration process. Although their results appear to disagree with the present observations, there may be not essential differences, because their experiment might be carried out solely on innervated melanophores. However, a direct comparison of both results is difficult due to the different material and method used.

Fujii and Novales (1968) showed that tetrodotoxin (TTX) failed to affect melanin movements in melanophores of *Fundulus heteroclitus* and of *Rana pipiens*. They presumed that electrogenic Na^+ channel were probably not involved in the response of the melanophores to MSH or catecholamines. Fingerman (1969) examined the effects of ouabain and TTX on the response of crustacean erythrophores to red pigment concentrating hormone. The response was progressively inhibited as the concentra-

tion of ouabain increased in the range of 10^{-6} to 10^{-3} g/ml, but it was enhanced by TTX.

On the physiological and biochemical roles of the membrane on pigment movements in fish melanophores further extensive studies are awaited.

Recently, it has been revealed that a possible action site of mersalyl is on the α adrenergic receptors of melanophores. The details will be published elsewhere.

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