Tetrodotoxin: Effects on Fish and Frog Melanophores

Abstract. *Tetrodotoxin blocks the response of killifish melanophores to electrical stimulation of their melanin-aggregating nerves. The direct response of killifish or frog melanophores to hormones is not affected. Since there is no effect on melanin aggregation resulting from the release of adrenergic neurotransmitter in killifish melanophores, we conclude that tetrodotoxin acts by blocking nerve conduction.*

Tetrodotoxin (TTX), the potent poison of puffer fishes and newts of the genus *Taricha*, has been widely used to study neuroeffector systems because of its highly specific blockage of action potentials (*1*). Thus, we chose TTX for study of the responses of vertebrate melanophores to nervous and hormonal stimuli, in order to learn more about the mechanisms of melanophore control. Melanophores are the melanin-bearing chromatophores largely responsible for the adaptive color changes exhibited by cold-blooded vertebrates. In teleost fishes they are controlled primarily by two sets of autonomic nerve fibers: melanin-aggregating (adrenergic) and melanin-dispersing (cholinergic?). In amphibians, they are mainly controlled by the pituitary melanocyte-stimulating hormone (MSH), which produces melanin dispersion (*2*).

The action of MSH on frog melanophores requires the presence of Na^+. This requirement is highly specific because no cation has yet been found to be capable of replacing Na^+ in permitting the response of frog (*Rana pipiens*) melanophores to MSH (*3*). Since TTX blocks the electrogenic Na^+ channels in the plasma membrane responsible for action potential production (*4*), it was used so that we might determine whether Na^+ enters the melanophore through such channels during their response to MSH.

For the fish experiments, the killifish, *Fundulus heteroclitus*, was used. A long strip from the caudal fin consisting of two fin rays and the web between them was isolated and split into two symmetrical halves. This "split preparation" (*5*) was held spread in a glass perfusion chamber on the stage of a Zeiss inverted microscope. Irrigation with physiological solution (*6*) usually caused full dispersion of melanin pigment in the melanophores. The light transmitted through a circular area of web of about 100 or 150 μ in diameter, just sufficient to contain a single melanophore, was introduced to a CdS photoconductive cell. Previously, a selenium photocell has been utilized for this purpose (*5, 7*). The changes in photoelectric current due to the melanophore response were recorded on a Varian model G-2022 paper recorder. All experiments were done at room temperature between 23° and 25°C.

Electrical stimulation of nerves was applied through a pair of platinum wires, 300 μ in diameter and insulated up to the tip, laid about 1 mm proximal to the melanophore to be observed. The electrodes were placed parallel to the fin ray, the distance between them being about 100 μ. Negative biphasic pulses were delivered in reference to the distal wire by means of a Grass model S4 stimulator. In the left part of Fig. 1A, the control response to nerve stimulation for 30 seconds is seen (10 pulses per second, 1-msec duration, strength 7.0 volts). A rapid increase in transmittance was recorded as a result of melanin aggregation within the melanophore. Although both the aggregating and dispersing fibers might have been stimulated, the response was apparently caused by the activation of the former.

The medium was then changed to

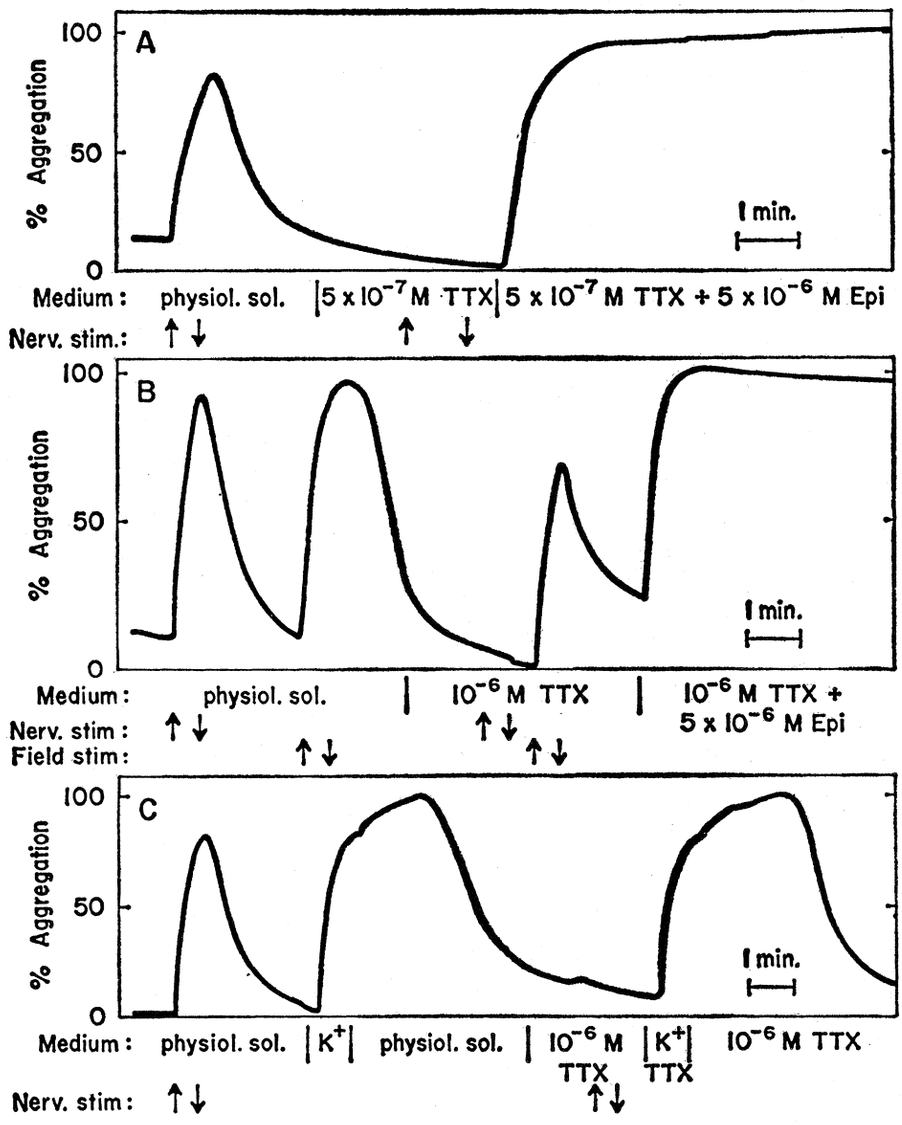

Fig. 1. Typical redrawn recordings of the effects of tetrodotoxin (TTX) on the melanin-aggregation response of *Fundulus* melanophores to various stimuli. The beginning of stimulation is indicated by an upward arrow and the end by a downward arrow. (A) The effect of nervous stimulation in the absence and presence of TTX. The effect of epinephrine (*Epi*) in the presence of TTX. (B) The effect of nervous and field stimulation in the absence and presence of TTX, followed by the effect of epinephrine in TTX. (C) The effect of nervous stimulation and potassium in the absence and presence of TTX.

physiological solution containing $5 \times 10^{-7}M$ TTX (Sankyo Co., Tokyo). Ninety seconds after the application of TTX, nervous stimulation was again applied for 60 seconds, but there was no response. We then applied $5 \times 10^{-6}M$ epinephrine in the physiological solution which contained the same concentration of TTX as the previous solution. A profound aggregation response was recorded. This is exactly what would have occurred in the absence of TTX, since it is well known that epinephrine or norepinephrine acts directly on fish melanophores and that the transmitter of the aggregating nerves may be an adrenergic one (*2*, *7*). Therefore, we can rather safely conclude that TTX has essentially no effects on melanophores through interference with pigment movements or with their responsiveness to directly acting substances or liberated transmitter. A series of similar experiments showed that TTX is still effective in blocking nervous stimulation at concentrations as low as 10^{-7} mole/liter, and that, at concentrations up to 10^{-5} mole/liter, there is no discernible inhibitory effect on epinephrine action.

Figure 1B is an example of the recordings showing the effect of TTX on the response of a melanophore to field stimulation. Such stimulation produces release of transmitter. The melanophore to be studied was positioned about halfway between two chloride-covered silver plates, arranged so as to form current lines vertical to the fin rays. The distance between electrodes was about 1 mm. Alternating current at 60 cycle/sec was used. The field stimulation of 2 volt/mm was effective in causing a rapid cellular response, just as the nervous volley did. However, after the application of $10^{-6}M$ TTX, field stimulation was still effective in producing the response, whereas nervous stimulation failed to induce pigment aggregation. Epinephrine was also effective in the presence of TTX.

Denervated melanophores (*5*) are unresponsive to an electrical field. Field stimulation is also ineffective when the preparation is treated with dibenamine, an adrenergic blocking agent (*7*), and when transmission from nerve to melanophore is blocked by eliminating alkaline-earth cations from the medium (*8*). Our observations, therefore, suggest that the responsivenes of the presynaptic membrane still remains after the application of TTX, and that, if a depolarizing stimulus such as an alternating field is applied, the transmitter substance can be released to produce the effector response. This phenomenon is analogous to that recently described by Katz and Miledi (*9*) on the motor nerve endings of the frog and giant synapses of the squid. They called it "functional isolation" of synapses by TTX.

Table 1. The effect of tetrodotoxin (TTX) on the response of frog skin melanophores to α-melanocyte-stimulating hormone (α-MSH). Darkening is expressed as the decrease in reflectance (Δ_{60}) in galvanometer units (GU) over a 60-minute period. Figures are means $\pm$ standard errors. Numbers of skins are in parentheses.

α-MSH (unit/ml)	TTX (mole/liter)	Δ_{60} (GU)
0	10^{-5}	$+1 \pm 2$ (2)
2	0	47 ± 4 (8)
2	10^{-5}	46 ± 4 (6)

Potassium ions induce pigment aggregation in fish melanophores (*10*). Recently, it was found the K^+ does not act directly on the melanophore itself, but on the presynaptic structure, resulting in release of transmitter which produces the response (*5*, *11*). We thought that we could confirm the mode of action of TTX on the melanophore nervous system by making use of this characteristic action of K^+. As expected, the response to K^+ (*12*) was not altered even after the addition of $10^{-6}M$ TTX (Fig. 1C), further supporting the concept that the presynaptic membrane is resistant to TTX. Fundamentally similar results were obtained with procaine, though much higher concentrations were required to produce axonal block (*8*).

We performed the experiments with amphibian melanophores, using the isolated skin technique developed by Shizume, Lerner, and Fitzpatrick (*13*). Dorsal thigh and leg skins were removed from pithed *Rana pipiens*, mounted on aluminum frames, and paled in Ringer solution rinses. Addition of MSH causes melanin dispersion. The resultant darkening was measured by the decrease in reflectance which takes place; the reflectance was measured with a Photovolt photoelectric reflection meter, model 610, calibrated with external standards. The decrease in reflectance is correlated with dispersion of melanin in the melanophores (*3*).

Tetrodotoxin had no effect on the response of frog melanophores to synthetic α-MSH (Table 1). Tetrodotoxin had no effect alone. Synthetic α-MSH caused marked darkening over a 1-hour period. Tetrodotoxin had no effect on the response to α-MSH at a concentration of $10^{-5}M$, which is greater than that required to block conduction in the frog sciatic nerve (*9*). The darkened skins were then treated with epinephrine at a concentration of $5 \times 10^{-5}M$. In all cases, complete paling occurred, even in the presence of $10^{-5}M$ TTX, as measured both by the decrease in reflectance and by microscopic examination. The average percentage of paling of four skins without TTX was 95 percent and of six skins in the presence of TTX was 97 percent. Furthermore, previous treatment of two skins with TTX for 90 minutes had no effect on their subsequent response to MSH.

These results suggest that TTX fails to affect melanin movements in melanophores. Furthermore, they show that electrogenic Na^+ channels are probably not involved in the response of melanophores to MSH or catecholamines. The lack of effect of TTX on melanophores is another example of the general lack of effect of TTX on the responses of effector cells to direct stimulation by pharmacological agents (*1*).

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6. Composition: 233 m*M* NaCl, 8.1 m*M* KCl, 2.3 m*M* $CaCl_2$, 3.7 m*M* $MgCl_2$, 5.5 m*M* glucose; *p*H 7.4 with 5.0 m*M* Tris-HCl buffer.
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