Effects of Tetrodotoxin on Excitability of Squid Giant Axons in Sodium-Free Media

Abstract. *The effect of tetrodotoxin on excitability of internally perfused squid giant axons immersed in various sodium-free media was examined. Action potentials were found to be suppressed by this substance, with or without sodium ion in the external medium. Tetrodotoxin showed a strong suppressive effect upon action potentials produced in media containing salts of only divalent cations ($CaCl_2$, $CaBr_2$, $SrCl_2$, or $BaCl_2$). Our findings concerning the action of tetrodotoxin do not support the separate-channel hypothesis for excitable membranes.*

Tetrodotoxin (TTX) is the potent poison found in both the Japanese pufferfish and the California newt (*Taricha torosa*) (*1*). In extremely low concentration (10^{-7} to 10^{-8} g/ml) tetrodotoxin suppresses action potentials in a variety of excitable tissues, including frog myelinated nerve fibers, frog muscle fibers (*2*), and lobster and squid giant axons (*3, 4*). Based on the current theory of nerve excitation, which proposes separate ion-specific channels, Narahashi *et al.* (*5*), Nakajima *et al.* (*6*), and Nakamura *et al.* (*4*) have proposed that TTX specifically blocks the "sodium channel," whereas the "potassium channel" remains unaffected. The evidence for this interpretation may be summarized as follows: (i) Delayed outward membrane current under voltage-clamp is not affected when the action potential is blocked by TTX (*3*). (This current is ascribed to a transport of ions through the "potassium channel.") (ii) This substance does not block the action potential produced in external media rich in potassium (*6*). (iii) It does not block the action potential in those tissues [for example, the muscle fibers of crustacea (*7*) or the taenia coli of the guinea pig (*8*)] in which excitability is maintained in media free of sodium.

We have examined whether or not the interpretation of the action of TTX stated above is applicable to squid giant axons under intracellular perfusion. Giant axons of the squid *Loligo pealii* are excitable in various external media in which sodium ions are replaced by other organic and inorganic univalent cations (*9*). After enzymatic removal of axoplasm and under continuous intracellular perfusion with dilute cesium-salt solutions, excitability of squid giant axons can also be maintained in external media containing salts of only divalent cations (that is, free of univalent cations) (*9*). [Although enzymatic digestion of axoplasm is not absolutely essential for excitability under these conditions, it greatly facilitates the maintenance of continuous intracellular perfusion. The requirements for excitability in media containing sodium substitutes or only divalent cations have been extensively described and discussed (*9*).] With these experimental conditions, the specificity attributed to the action of TTX can be directly examined. If it is assumed that the effect of TTX is specific for the sodium ion, action potentials in sodium-free media should not be blocked by TTX. But, if TTX is specific for the "sodium channel," those ion-species that pass through the channel could be determined by TTX blockage.

The method of intracellular perfusion has been described in detail (*9*). We prepared the perfusion fluid by mixing a 600-m*M* CsF solution with 12 percent (by volume) glycerol to a final concentration of 25 to 100 m*M* CsF. Cesium phosphate buffer was used to adjust the *p*H to the range between 7.1 and 7.4. For the experiments described in Fig. 1D and Fig. 2, axoplasm in the perfusion zone was initially removed by enzymatic digestion with a protease (Pronase, Prozyme, or Nagarse, 0.1 mg/ml). These preparations were first perfused intracellularly with the enzyme-containing solution for approximately 1.5 minutes and then with enzyme-free CsF solution.

An enameled silver wire (50 μ in diameter) with a 20-mm-long uninsulated portion at the end was used for uniform internal stimulation of the entire perfusion zone (10 to 15 mm in length). Stimulating currents were obtained from a Tektronix pulse generator, usually through a 5-Mohm resistor. A large silver wire immersed in the external medium served as the ground electrode. The internal recording electrode was an enameled silver wire (50 μ in diameter) with a 1-mm-long uninsulated portion placed in the center of the internal perfusion zone. The recording and the stimulating electrodes were introduced into the perfused zone of the axon interior through the outlet cannula.

Tetrodotoxin (Sankyo Co.) was mixed with the extracellular medium or with distilled water (10^{-6} to 10^{-5} g/ml), and 50 μl of this solution was added to the external media in the

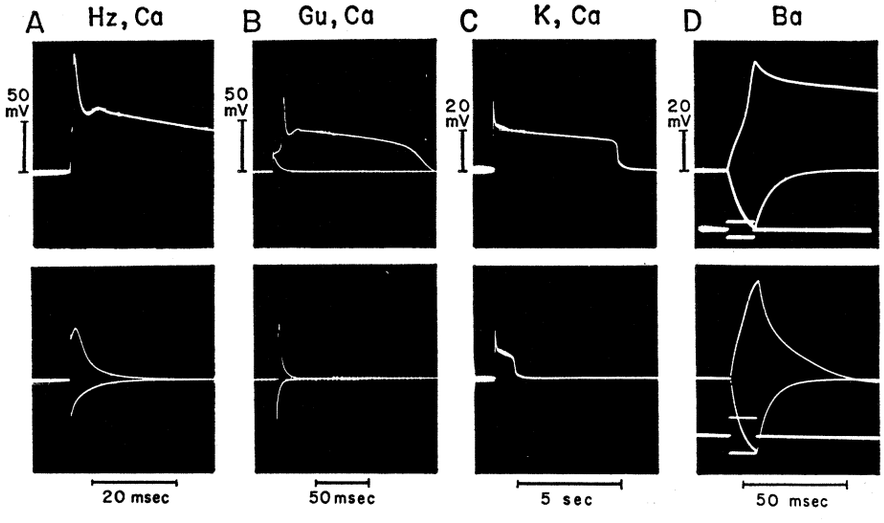

Fig. 1. Effect of TTX on action potentials of perfused squid axons in several sodium-free media. Records from four different axons. Upper records were taken before application of TTX; lower records, after application of TTX. (A) Outside medium contained 300 m*M* hydrazinium chloride and 200 m*M* $CaCl_2$, *p*H 7.7; inside medium, 90 m*M* CsF and 10 m*M* cesium phosphate, *p*H 7.3; TTX concentration, 2×10^{-8} g/ml. (B) Outside, 100 m*M* guanidinium chloride, 200 m*M* TMA chloride, and 200 m*M* $CaCl_2$, *p*H 8.0; inside, same as in (A); TTX concentration, 2×10^{-8} g/ml. (C) Outside, 10 m*M* KCl, 290 m*M* TMA chloride, 200 m*M* $CaCl_2$, *p*H 8.0; inside, same as in A; TTX concentration, 4×10^{-7} g/ml. (D) Outside, 200 m*M* $BaCl_2$, *p*H 7.9; inside, 22.5 m*M* CsF and 2.5 m*M* cesium phosphate, *p*H 7.4; TTX concentration, 2×10^{-7} g/ml. In (D), the upper oscillograph beam represents the membrane potential; the lower beam, the current intensity; two sweeps with outward and inward current pulses are superimposed.

Lucite chamber in which the perfused axon was mounted. The total quantity of the external solution was measured after the experiment, and the final concentration of TTX was determined.

The production of action potentials in various sodium-free media was strongly suppressed by application of TTX in a concentration between 2×10^{-8} and 4×10^{-7} g/ml (Fig. 1). Action potentials were produced by axons immersed in media containing hydrazine (Fig. 1A); guanidine (Fig. 1B); hydroxylamine, ammonium, sodium, or potassium (Fig. 1C). The optimum concentrations of these cations for producing action potentials differed according to the ionic species. We varied the concentration of these cations by using tetramethylammonium (TMA) chloride, maintaining the total univalent cation concentration at 300 m*M*. All solutions contained 200 m*M* calcium chloride. Under the action of TTX, the electric responses of the axons in sodium-free media always became shorter, smaller, and graded within 1 to 5 minutes. Only poor recovery could be obtained when the preparations were washed for 10 minutes with solutions free of TTX.

Susceptibility to the blocking action of TTX varied with the ionic species in the external media. Action potentials produced in external media containing 10 m*M* potassium ions were most resistant to the blocking action of TTX (Fig. 1C). When 4×10^{-7} g/ml of TTX was applied, the action potential became smaller in amplitude and shorter in duration, but it remained all-or-none in nature. This is in agreement with observations made by Nakajima *et al.* (*6*). In our experiment, however, the action potential was clearly suppressed in the concentration range of TTX used. Thus, the difference in effects is quantitative rather than qualitative.

Tetrodotoxin was also effective in blocking action potentials produced in external media containing salts of only divalent cations (Fig. 1D). The external medium contained 200 m*M* barium chloride and glycerol. After application of TTX (2×10^{-7} g/ml), the action potential fell rapidly, and only a small local response remained. The concentration of TTX used in this particular experiment was relatively high; usually 2×10^{-8} g/ml was sufficient to block the action potential. Similar blocking action was observed on action potentials produced in media containing $CaCl_2$, $CaBr_2$, or $SrBr_2$, with no external univalent cations present.

The susceptibility of action potentials to TTX in media containing only $CaCl_2$ and in those with NaCl and $CaCl_2$ was compared (Fig. 2). The external medium first contained 200 m*M* $CaCl_2$ and glycerol (Fig. 2, A and B). The action potential was promptly blocked when 50 μl of a solution containing 2×10^{-6} g of TTX per milliliter was added (Fig. 2C); the final concentration of TTX was 3.3×10^{-8} g/ml. The ability of this axon to produce all-or-none action potentials was restored when 0.6 ml of 600 m*M* NaCl was added to the external medium (Fig. 2D). The final sodium concentration was 97 m*M*. Although the TTX concentration was decreased to 2.5×10^{-8} g/ml, this decrease apparently did not cause recovery; this concentration was usually more than enough to block action potentials in sodium-free media, and recovery after rinses with TTX-free solutions was usually very poor.

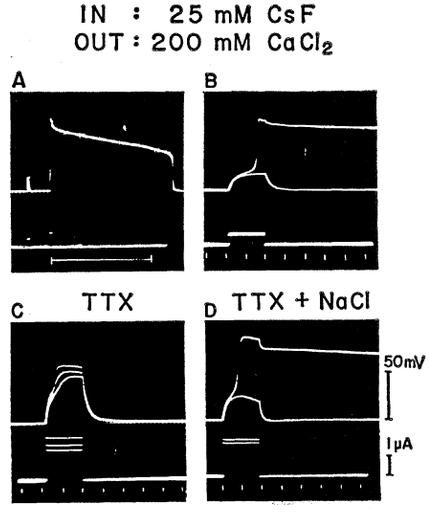

Fig. 2. Recovery of excitability, after addition of NaCl to the outside medium, in an axon poisoned with TTX. All records were taken from the same axon intracellularly perfused with 25 m*M* CsF (*p*H adjusted to 7.3 by addition of a small amount of cesium phosphate buffer). The upper beam represents membrane potential; the lower beam, membrane current. (A) An action potential in 200-m*M* $CaCl_2$ solution. (Note that the first stimulus was subthreshold.) Time marker, 5 seconds. (B) Same as in A but on a rapid time base. Two sweeps at two slightly different stimulus strengths are superimposed. Dots are 50 msec apart. (C) After addition of TTX with a final concentration of 3.3×10^{-8} g/ml. Three sweeps are superimposed. Time base as in B. (D) After addition of 0.6 ml of 0.6*M* NaCl. Two sweeps with different stimulus strengths are superimposed.

Recovery could also be produced by the addition of a small amount of crystalline NaCl. In these experiments, the final concentration of sodium ions was about 50 m*M*, and the concentration of TTX in the medium did not change. (Note that the osmotic pressure of the solution increased about 10 percent, an insignificant change in these qualitative experiments.)

Our observations indicate that the action of TTX on the membrane of the squid giant axon is suppression of excitability in media with and without sodium ions. As this effect is invariably observed for various external ionic compositions, it is obvious that TTX does not have any specificity for the sodium ion. This conclusion seems to be in agreement with observations of Moore and Narahashi (*10*), who showed that TTX effectively blocks the action potentials of squid giant axons produced in "lithium sea water."

It has been argued that TTX may be specific for the "sodium channel," rather than for the sodium ion (*10*). It seems difficult to explain our findings, if one assumes such channel specificity of TTX, for the following reasons:

1) If suppression of excitability by TTX is assumed to result from blockage of the "sodium channel," our findings have to be interpreted as indicating that a variety of cations (such as Ba, Sr, Ca, K, NH_4, hydrazinium, and so forth) are transported through the "sodium channel." In this case, one has to abandon the assumption that there is a channel specific for sodium ions in the axon membrane.

2) According to the current theory of excitation (*11*), each ion channel must have its own well-defined electromotive force. The electromotive force for a channel is usually calculated by use of either the Nernst equation (*11*) or the constant-field equation (*12*). However, calculation with the Nernst equation is not possible when the ion concentration on one side of the membrane is zero. Furthermore, calculation with the constant-field equation is impossible when one takes divalent ions into consideration. Therefore, it is difficult to define the electromotive force of the channel upon which TTX is assumed to act. Since the behavior of the axonal membrane must fol-

low standard physiochemical principles (*13*), it is not necessary to postulate special channels to interpret these results (*14*).

3) The concept of "specific blocking action of the sodium-carrier system" arose partly from the observed difference between the effect of TTX on action potentials produced in Ringer's (sodium-rich) solution and that in potassium-rich media (*4*). Although in the squid giant axon the effectiveness of TTX blockage differs according to the composition of the external media, the difference is only quantitative. Our experiments indicate that, at certain concentrations of TTX in the external medium, the action of TTX could be called "calcium-specific" (Fig. 2). However, this would obviously be inappropriate, since an increase in the concentration of TTX can also block the action potential in media containing sodium.

Our findings concerning the effects of TTX on squid giant axons do not support the separate channel hypothesis for excitable membranes.

There is considerable evidence indicating that exchange of cations at sites of the membrane macromolecules is the primary physicochemical event leading to the process of excitation (*14*). Strong binding of TTX to the charged sites on the external layer of membrane macromolecules would hinder the rapid exchange of small univalent and divalent cations, and would account for the observed effects of TTX. This explanation is consistent with the fact that TTX when applied intracellularly has no effect on the action potential (*15*).

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Negro Variant of Glucose-6-Phosphate Dehydrogenase Deficiency (A^-) in Man

Abstract. *Glucose-6-phosphate dehydrogenase in erythrocytes of the Negro type associated with enzyme deficiency (A^-) was separated by chromatography on a carboxymethyl-Sephadex column from the electrophoretically indistinguishable Negro variant with normal enzyme activity (A^+). Quantitative immunologic neutralization tests indicated that the A^- enzyme had about the same enzymatic and serological activity as the A^+ and the normal (B^+) enzymes. The enzyme activity of the A^- variant in young erythrocytes was similar to that in young cells from normal individuals, although the activity of the A^- variant in unfractionated red cells was 10 to 15 percent of normal. These data indicate that the basic defect in the variant enzyme (A^-) is a structural mutation which causes more rapid degradation of the enzyme during erythrocyte aging.*

The erythrocytes of about 20 percent of Negro males have an electrophoretically rapid variant of glucose-6-phosphate dehydrogenase with normal enzyme activity (A^+). The cells of another 10 to 15 percent have a glucose-6-phosphate dehydrogenase which is electrophoretically indistinguishable from A^+ but has reduced (10 to 15 percent of normal) activity (A^-) (*1*). The levels of enzyme in nucleated tissues such as leukocytes and liver of A^- Negro males are normal or only slightly decreased (*2*). No consistent differences have been found between A^+ and A^- enzymes in enzymatic properties and immunological properties (*3*). Kirkman *et al.* suggested that enzyme deficiency in erythrocytes may be caused by a quantitative reduction in the number of the enzyme molecules (*4*). Luzzato and Allan observed differences in heat stability and elution patterns on diethylaminoethyl (DEAE)-Sephadex column chromatography between the A^+ and A^- enzymes (*5*).

Table 1. Enzyme activities in red cells of different age groups. The values are mean values and ranges (parentheses) of the number of units of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity per gram of hemoglobin in red cells obtained from three A^- and three normal (B^+) individuals.

Glucose-6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase	
Normal	A^- deficiency	Normal	A^- deficiency
Younger cells			
17 (16.1–18.2)	13 (12–15)	8.7 (7.0–10.2)	8.1 (6.5–10.5)
Total cells			
6.6 (4.4–7.8)	0.93 (0.76–1.1)	3.8 (3.3–4.2)	4.0 (2.7–5.1)
Older cells			
4.1 (3.4–4.9)	0.60 (0.4–0.7)	3.1 (2.8–3.4)	3.7 (2.5–4.6)

To find out whether the low activity in the red cells deficient in glucose-6-phosphate dehydrogenase from Negro males resulted from a structural abnormality producing a less-active enzyme molecule, from decreased rate of production, from an increased degradation rate, or from a combination of these effects, the following experiments were carried out.

The A^+ and A^- enzymes can be separated by DEAE-Sephadex column chromatography (*5*). The low recovery of the activity of A^- enzyme from the column could also be confirmed. Since the A^- enzyme was inactivated in the absence of nicotinamide-adenine dinucleotide phosphate (NADP), the low recovery of A^- enzyme from the DEAE-Sephadex column may have been partly due to loss of NADP by the anion exchanger during chromatography. Another chromatographic system—carboxymethyl cellulose–Sephadex column—gave a more effective separation of the A^+ and A^- enzymes (Fig. 1). The recovery of enzyme activity was better than 90 percent for each enzyme. These findings indicate structural differences between the A^+ and A^- enzymes.

Rabbit antiserum was prepared (*7*) by a series of subcutaneous injections