Tetrodotoxin Blockage of Sodium Conductance Increase in Lobster Giant Axons

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ABSTRACT Previous studies suggested that tetrodotoxin, a poison from the puffer fish, blocks conduction of nerve and muscle through its rather selective inhibition of the sodium-carrying mechanism. In order to verify this hypothesis, observations have been made of sodium and potassium currents in the lobster giant axons treated with tetrodotoxin by means of the sucrose-gap voltage-clamp technique. Tetrodotoxin at concentrations of 1×10^{-7} to 5×10^{-9} gm/ml blocked the action potential but had no effect on the resting potential. Partial or complete recovery might have occurred on washing with normal medium. The increase in sodium conductance normally occurring upon depolarization was very effectively suppressed when the action potential was blocked after tetrodotoxin, while the delayed increase in potassium conductance underwent no change. It is concluded that tetrodotoxin, at very low concentrations, blocks the action potential production through its selective inhibition of the sodium-carrying mechanism while keeping the potassium-carrying mechanism intact.

Tetrodotoxin is the active principle of the puffer poison which has long been a matter of interest because the puffer fish is one of the major sea foods in Japan. It blocks peripheral nerve and muscle conduction as well as the central nervous system at very low concentrations (Fleisher, Killos, and Harrison, 1961; Iwakawa and Kimura, 1922; Kuga, 1958; Kuriaki and Wada, 1957; Kurose, 1943; Matsumura and Yamamoto, 1954; Murtha, 1960; Wada, 1957; Yano, 1938). This toxin mimics local anesthetics such as cocaine and procaine in that block is not accompanied by a change in resting potential (Furukawa, Sasaoka, and Hosoya, 1959; Nakajima, Iwasaki, and Obata, 1962; Narahashi, Deguchi, Urakawa, and Ohkubo, 1960; Shanes, 1958).

It seems reasonable to assume that the primary action of local anesthetics

is not on the mechanism that determines resting potential but on the mechanism that produces action potentials. Several attempts have so far been made to verify the validity of this assumption. Observations of the action potential configuration, the resting potential, and the resting membrane resistance in nerve, muscle, and Purkinje fibers have led to the hypothesis that cocaine, urethane, and several other anesthetics affect the system which is responsible for carrying sodium ions through the membrane (Inoue and Frank, 1962; Narahashi, 1964 *a*; Straub, 1956; Thesleff, 1956; Weidmann, 1955). However, the most convincing evidence was provided by the voltage-clamp experiments with squid giant axons in which cocaine and procaine were shown to suppress the rise of sodium and potassium conductances normally occurring upon depolarization (Shanes, Freygang, Grundfest, and Amatniek, 1959; Taylor, 1959). Also in voltage-clamp experiments with the nerve cells of a marine pulmonate mollusc, *Onchidium*, urethane was found to block the initial inward current without appreciably changing the final steady current (Hagiwara and Saito, 1959).

From observations of the action potential and some other membrane characteristics of muscle fibers, it has been suggested that tetrodotoxin blocks excitability through its selective inhibition of the sodium-carrying system without affecting the potassium-carrying system (Nakajima *et al.*, 1962; Narahashi *et al.*, 1960). This view was further supported by the finding with lobster giant axons that the maximum rate of rise of the action potential, which is indicative of the inward sodium current during activity, is decreased much faster than is the rate of fall during the course of tetrodotoxin block (Narahashi, 1964 *a*). The present voltage-clamp study was undertaken in order to gain more critical and conclusive evidence for or against this view.

METHODS

Giant axons in the circumesophageal connectives of the lobster, *Homarus americanus*, were used throughout the experiments. The voltage-clamp experiments were performed in a sucrose-gap chamber, the method being essentially the same as that described by Julian, Moore, and Goldman (1962 *a*, *b*). Two sucrose streams electrically isolated three regions of an axon. The very short central portion (50 μ or less) was bathed in sea water and called an "artificial node." The membrane potential of this node was measured as the potential difference between the central pool and one end of the axon in KCl. Stimulating or clamping current was injected *via* the other end, also in KCl. The tetrodotoxin was added to the sea water bathing the outside of the node. All membrane potentials (E_M) were expressed on an absolute scale as internal potential minus external potential.

The flowing sucrose caused the node to be hyperpolarized by some 20 to 70 mv. Thus, the resting potential (E_R) of the node was measured to be -90 to -140 mv, compared with the typical value of $E_R = -70$ mv measured with micropipettes in other preparations. In order to remove possible inactivation of the sodium-carrying

system in the resting state (Hodgkin and Huxley, 1952 *c*), the steady membrane potential on which clamping voltage pulses were superimposed, *i.e.* the "holding potential" (E_H), was kept hyperpolarized by 10 to 30 mv or at a value of −100 to −170 mv.

The ionic compositions (mM) of the artificial sea water around the node and of the end pool solutions were as follows:—

	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	SO^{--}_4	HCO^-_3	pH
Sea water	468	10	25	8	533	4	2.5	7.8
KCl solution	0	478	25	8	533	4	2.5	7.8

The sucrose solution was isotonic and contained 725 mM sucrose (Julian *et al.*, 1962 *a*). The tetrodotoxin used was a crystalline preparation having a median lethal dose of 0.01 μg/gm for the mouse (Sankyo Company Ltd., Tokyo). The experiments were conducted at a temperature of about 10°C.

RESULTS

In accordance with the observations by Julian *et al.* (1962 *a*), there occurred a marked hyperpolarization upon introducing the isotonic sucrose solution to the chamber. Therefore, the apparent resting potential measured after introducing the KCl solution into the potential reference pool was very high (E_R = −90 to −140 mv). Thus, action potentials were observed as high as 170 mv in magnitude.

Tetrodotoxin at a concentration of 1×10^{-7} gm/ml abolished the action potential very quickly without changing the resting potential. Although the exact time needed for blockage was not measured, the action potential disappeared as soon as the dead space in the solution flow system had been cleared (within 1 minute). The toxin was effective in blocking excitability even at a concentration of 5×10^{-9} gm/ml, but it took as long as 4 to 8 minutes to do so. Recovery after washing was incomplete or absent in most cases. However, the fact that complete recovery was obtained in a few cases indicates that the action of tetrodotoxin is reversible under appropriate conditions. A previous study has clearly shown that this is true when whole preparation is washed with sea water (Narahashi, 1964 *a*).

Fig. 1 shows an example of a series of records of membrane current under voltage-clamp conditions and of action potential. On passing a brief pulse (0.5 to 1.0 millisecond) of cathodal or outward current through the unclamped membrane, there occurred a depolarization which attained nearly 100 mv without producing a regenerative action potential. When the depolarization reached a certain level, an action potential was generated (Fig. 1A, top record). In this particular case, the action potential arose at the termination of the cathodal pulse, which is seen as a notch on the record. The critical depolarization for firing was then higher than that measured by means

of the ordinary micropipette method (30 to 40 mv). This is attributable to the hyperpolarization by sucrose, because, on anodal hyperpolarization, the threshold potential or the membrane potential at which firing occurs, is kept unchanged at $E_M = -30$ to -40 mv (Narahashi, 1964 *b*).

When the membrane was clamped at a depolarized level of about $E_M = -40$ to -50 mv, there appeared, following the initial capacitative surge, a transient inward current which was followed by an outward steady current (Fig. 1A). Both currents were increased in magnitude with greater depolarization, *i.e.*, with smaller membrane potential. With further increasing de-

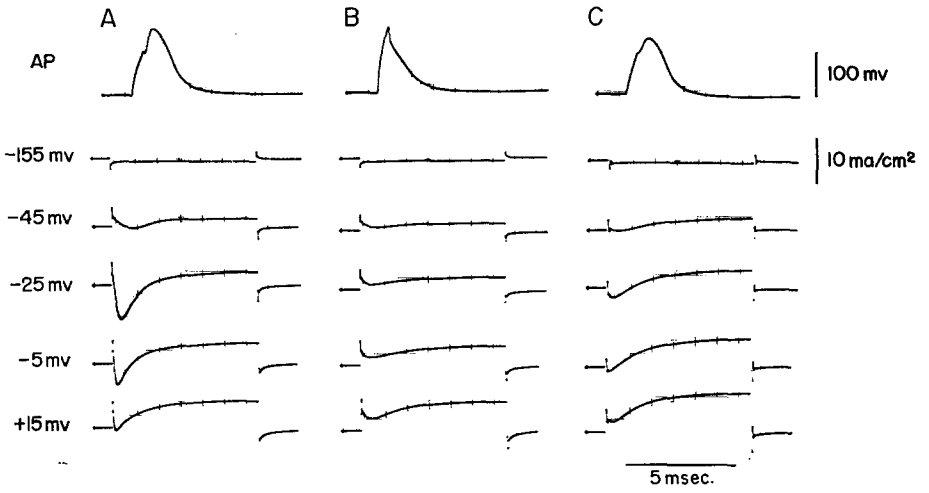

FIGURE 1. Records of the action potential (*AP*) and the membrane current at various membrane potentials before (column A) and during (column B) treatment with tetrodotoxin 3×10^{-8} gm/ml, and after washing with normal sea water (column C). The values of the clamped membrane potentials (inside measured with respect to outside), which apply to all columns, are shown at extreme left. Preparation 116-Af.

polarization, although the delayed outward current was increased steadily, the transient inward current was decreased again and finally converted into an outward current at around $E_M = +35$ mv. It is then assumed from these observations that the transient inward current is carried by sodium ions and the delayed outward current by potassium ions (Hodgkin and Huxley, 1952 *a*, *b*; Hodgkin, Huxley, and Katz, 1952; Julian *et al.*, 1962 *b*).

Application of tetrodotoxin at a concentration of 3×10^{-8} gm/ml blocked the action potential (Fig. 1B, top record). The inflection on the falling phase shows a residual activity which failed to become regenerative. Under the clamped conditions, the sodium current was suppressed while the potassium current was almost unchanged (Fig. 1B). Washing with normal sea water

brought about partial recovery both in sodium current and in action potential (Fig. 1C).

Current-voltage relations are illustrated in Fig. 2. There was a small leakage current (I_L) which changed linearly over the range of $E_M = -160$ mv to -60 mv. A linear extrapolation of I_L was assumed, and subtracted from each record of membrane current (I_M). The peak sodium current (I_{Na}) was then estimated as the peak value of the early transient of $I_M - I_L$,

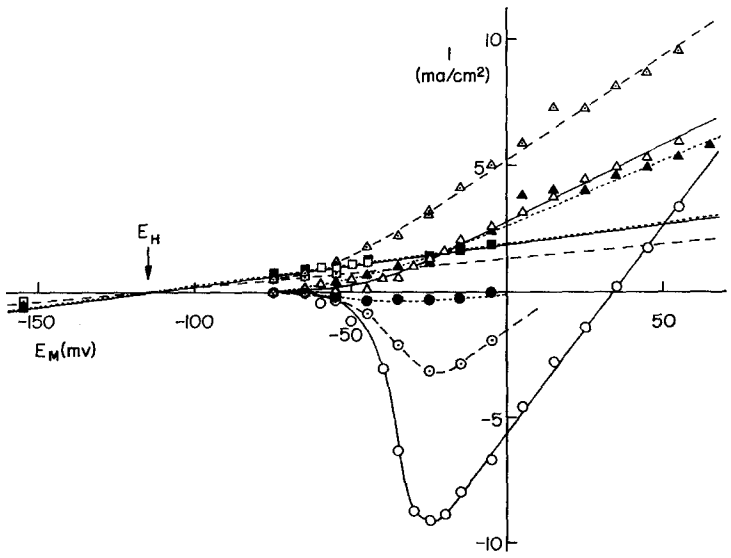

FIGURE 2. Current-voltage relations before (open symbols and solid lines) and during (filled symbols and dotted lines) treatment with tetrodotoxin 3×10^{-8} gm/ml, and after washing with normal sea water (open symbols with dots and broken lines). Circles refer to peak sodium current corrected for leakage current, triangles refer to steady-state potassium current corrected for leakage current, and squares refer to leakage current. I, designated component of the membrane current (inward direction negative); E_M, membrane potential; E_H, holding potential. Preparation 116-Af.

and the steady-state potassium current (I_K) was taken as the final value of $I_M - I_L$ (Hodgkin and Huxley, 1952 *a*). From these current-voltage relations it is clear that tetrodotoxin suppresses the sodium current without any appreciable effect on the potassium current. Although the sodium current was partially restored after washing with normal sea water, the potassium current became larger in this particular case. The latter change was not always observed as is described below (*cf.* Table I).

The maximum sodium conductance (g_{Na}) was computed from the relation, $g_{Na} = I_{Na}/(E_M - E_{Na})$ (Hodgkin and Huxley, 1952 *a*, *b*), where E_{Na} refers to the membrane potential at which I_{Na} reverses its direction; *i.e.*, the sodium equilibrium potential. The maximum potassium conductance (g'_K) was taken

from the steady-state linear slope of $I_K - E_M$ curves. Thus, g'_K is not the potassium conductance defined as $g_K = I_K/(E_M - E_K)$ (Hodgkin and Huxley, 1952 *a*; *b*) where E_K refers to the potassium equilibrium potential, but the steady-state slope conductance defined as $\partial I_K/\partial E_M$ (Hodgkin and Huxley, 1952 *a*; Shanes *et al.*, 1959). The calculated conductances are summarized in Table I.

TABLE I

EFFECT OF TETRODOTOXIN ON MEMBRANE CONDUCTANCES

Concentration of tetrodotoxin	Preparation	Maximum sodium conductance g_{Na}					Maximum potassium conductance g'_K					Leakage conductance g_L				
		C	*T*	*R*	*T/C*	*R/C*	*C*	*T*	*R*	*T/C*	*R/C*	*C*	*T*	*R*	*T/C*	*R/C*
gm/ml		*mmho/cm²*					*mmho/cm²*					*mmho/cm²*				
	103-Ba	212	0		0		53	37		0.69		6	10		1.66	
1×10^{-7}	104-Ac	510	0	60	0	0.117	95	125	75	1.31	0.78	25	47	75	1.88	3.00
	104-Ad	325	0		0		90	95		1.05		50	62		1.24	
	Mean				0	0.117				1.01	0.78				1.59	3.00
	104-Bb	155	0	145	0	0.935	45	60	100	1.33	2.22	23	27	27	1.17	1.17
	104-Bd	345	75		0.217		40	110		2.75		62	70		1.20	
	115-Ba	300	20		0.066		75	50		0.66		17	19		1.11	
	115-Bb	180	35	85	0.194	0.472	60	50	35	0.83	0.58	15	20	24	1.33	1.60
3×10^{-8}	116-Ab	410	0		0		95	75		0.78		30	30		1.00	
	116-Ac	170	0		0		45	47		1.04		27	30		1.11	
	116-Af	165	10	95	0.060	0.575	62	53	85	0.85	1.37	17	17	11	1.00	0.64
	116-Ag	115	0		0		52	90		1.73		19	13		0.68	
	116-Bc	175	0		0		52	52		1.00		23	31		1.34	
	Mean				0.059	0.660				1.21	1.39				1.10	1.13
	114-Ab	300	95		0.316		85	85		1.00		18	24		1.33	
	114-Ac	200	80		0.400		67	82		1.22		22	25		1.30	
1×10^{-8}	114-Ad	197	55	105	0.279	0.532	87	90	115	1.03	1.32	23	35	35	1.52	1.52
	114-Ae	290	75	130	0.258	0.448	100	80	85	0.80	0.85	19	26	27	1.36	1.42
	Mean				0.313	0.224				1.01	1.08				1.37	1.47
	117-Ab	560	37		0.066		110	185		1.68		16	21		1.31	
	117-Ac	285	10		0.035		75	75		1.00		15	13		0.86	
	117-Ba	200	25		0.125		62	100		1.61		23	31		1.34	
5×10^{-9}	117-Bb	225	12		0.053		110	115		1.04		17	13		0.76	
	117-Bc	175	50		0.285		55	52		0.94		18	18		1.00	
	117-Bd	250	5		0.020		80	80		1.00		16	15		0.93	
	117-Be	275	11		0.040		55	45		0.81		6	6		1.00	
	Mean				0.089					1.15					1.02	
Over-all mean					0.115	0.333				1.09	1.08				1.27	1.86

C, control in normal sea water.
T, treatment with tetrodotoxin.
R, recovery after washing with normal sea water.

The sodium conductance decreased to very low values after exposing the axon to tetrodotoxin except for one experiment at a concentration of 1×10^{-8} gm/ml in which the decrease was less. The fluctuation of the percentage inhibition of the sodium conductance seems to be attributable to the fact that the decrease in the height of the action potential at the time of the voltage-clamp run was not the same in each preparation. At concentrations of 1×10^{-7} and 3×10^{-8} gm/ml, where the sodium conductance was reduced to very low values or to zero, the voltage-clamp measurements were made immediately after the action potential had been nearly completely abolished (such as seen in Fig. 1B); *i.e.*, 1 to 2 minutes after exposing the axon to the tetrodotoxin. On the contrary, in weaker concentrations of tetrodotoxin (1×10^{-8} or 5×10^{-9} gm/ml), the voltage-clamp measurements were started before the action potential had been abolished completely, the starting time ranging from 1 to 8 minutes after exposure to tetrodotoxin.

In contrast to this, the potassium conductance underwent little change during exposure to tetrodotoxin and after washing with normal sea water. There was a tendency for the leakage conductance (g_L) to increase after treatment with tetrodotoxin, but the change may have been due to deterioration with time, because g_L continued to rise after washing with normal sea water.

No attempt was made to correlate the spike height with the maximum sodium conductance in the tetrodotoxin-treated axons. The reason for this lies in two difficulties which must be overcome before the quantitative correlation becomes feasible: (*a*) It is difficult to maintain the spike height at a constant depressed level in tetrodotoxin. Although it took only 1 minute or less to complete one series of the voltage-clamp measurements, the spike height after a voltage-clamp run was usually found to be less than that before the run. (*b*) The amplitude from the resting level to the spike peak cannot be regarded as a reliable indication of the magnitude of the action potential when the spike is small, because the action potential is superimposed on the falling phase of the catelectrotonic potential which is larger in amplitude due to hyperpolarization by sucrose. The increase in membrane conductance during the spike is presumably less than normal when the spike height is smaller, making the error greater in the action potential measurement. All that can be said is that the maximum sodium conductance is greatly depressed when the action potential is abolished by treatment with tetrodotoxin.

DISCUSSION

This study shows clearly that tetrodotoxin blocks the mechanism for sodium conductance rise without affecting the mechanism for potassium conductance rise. This has been predicted on the basis of the observations that in tetrodotoxin, (*a*) the maximum rate of rise of the action potential, which is indicative of the peak inward current, is decreased more than is the rate of fall

(Narahashi, 1964 *a*), (*b*) no change occurs in the resting potential or resting membrane resistance, or in the delayed rectification, which is attributed to the delayed rise in potassium conductance upon depolarization (Nakajima *et al.*, 1962; Narahashi, 1964 *a*; Narahashi *et al.*, 1960).

Cocaine and procaine differ from tetrodotoxin in that they reduce not only the sodium current but also the potassium current (Shanes *et al.*, 1959; Taylor, 1959). Such concomitant actions on both mechanisms are observed with varying concentrations of calcium (Frankenhaeuser and Hodgkin, 1957; Shanes *et al.*, 1959). These findings have led to the suggestion that the increases in conductances to sodium and potassium are not as different in mechanism as one might infer from their different time courses (Shanes *et al.*, 1959). The present experiments do not favor this view but rather indicate that the sodium mechanism and the potassium mechanism are essentially different in nature. It is within the range of possibility that cocaine and procaine happen to have an affinity for both sodium and potassium channels in the membrane which are different in many respects.

Another example of the selective inhibition of the sodium-carrying system has been obtained with urethane in voltage-clamp experiments (Hagiwara and Saito, 1959). *N*-Butanol and *N*-octanol are assumed to block the sodium mechanism rather selectively, for the maximum rate of rise of the action potential is decreased more than is the rate of fall without accompanying appreciable change in resting potential (Narahashi, 1964 *a*). Although we cannot rely much upon the comparison of the rising and falling phases of the action potential to draw a final conclusion in this matter, the available data on tetrodotoxin, cocaine, and procaine clearly show that a parallel relation exists between the actions on the rising and falling phases and those on the sodium and potassium mechanisms (Narahashi, 1964 *a*; Shanes *et al.*, 1959; Taylor, 1959).

Another important aspect of the tetrodotoxin action is that the selective blockage of the sodium mechanism is brought about by very weak concentrations, the threshold concentration being about 5×10^{-9} gm/ml or less. Thus cocaine and procaine differ from tetrodotoxin not only in their nonselective blockage of both the sodium and the potassium mechanisms but also in their rather higher effective concentrations, which are around 10^{-3} to 10^{-4} gm/ml (Inoue and Frank, 1962; Narahashi, 1964 *a*; Shanes *et al.*, 1959; Taylor, 1959). Such strong and selective action of tetrodotoxin encourages us to explore further its mechanism in terms of the hypothesis that tetrodotoxin molecules bind with the membrane components in a way that prevents membrane calcium from being displaced by depolarization (Narahashi, 1964 *a*; *cf.* Feinstein, 1963).

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