Basis of Tetrodotoxin's Selectivity in Blockage of Squid Axons

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ABSTRACT The blockage of nerve activity by tetrodotoxin is unusually potent and specific. Our experiments were designed to distinguish whether its specificity of action was based on the identification of ions, the direction of cation flow, or differences in the early transient and late steady conductance pathways. Alkali cations were substituted for sodium in the sea water, bathing an "artificial node" in a voltage-clamped squid axon. When tetrodotoxin was added to the artificial sea waters at a concentration of 100 to 150 nM, it was found to always block the flow of cations through the early transient channel, both inward and outward, but it never blocked the flow of ions using the late steady pathway. We conclude that the selectivity of tetrodotoxin is based on some difference in these two channels.

It is now well established that tetrodotoxin (hereafter to be called TTX), the neurotoxin extracted from puffer fish,[1] blocks nerve impulse transmission by selectively interfering with the early transient conductance increase for sodium (Narahashi, Moore, and Scott, 1964; Nakamura, Nakajima, and Grundfest, 1965; Moore, 1965; Takata, Moore, Kao, and Fuhrman, 1966). TTX affects neither the late steady conductance changes, nor the kinetics of either the early or late conductance changes (Takata et al., 1966).

The present study was initiated in order to reevaluate the effect of TTX on the early transient current during large membrane potential steps because of some differences in results between lobster and squid axons when the axoplasm was stepped to potentials more positive than the sodium equilibrium potential E_{Na}. In unpublished experiments by one of us (MPB), it appeared that the outflow of sodium current which normally occurs in this potential range was not very effectively blocked. In most of the earlier experiments

[1] An identical toxin has been extracted from California salamanders and originally called tarichatoxin (Mosher, Fuhrman, Buchwald, and Fischer, 1964).

(e.g., Narahashi et al., 1964) no equivalent data were available. In one limited set of data on squid axon (Moore, 1965), there appeared to be an equal reduction of the sodium conductance at membrane potentials above and below E_{Na}.

In planning these studies, the following questions arose: (*a*) Does TTX selectively block the sodium conductance; i.e., is its action due to a direct discrimination against this ion per se? (*b*) Or, is its effect a result of its ability to block ions moving in a particular direction; e.g., does it block ions moving inward (most inward current is carried by Na) while sparing ions moving outward (usually K)? (*c*) Or, is its action based on an ability to distinguish between a pathway for the early transient current and one for the late steady current?

These three possibilities may not be mutually exclusive nor all inclusive. For example, the mode of TTX action could be a combination of two or more of these possibilities. However, the experiments were designed to help distinguish between these possible modes of TTX action. Various monovalent cations were substituted for sodium in the bathing sea water and the effect of TTX on voltage-clamped axon membranes observed.

A preliminary report of this work has been given (Moore, Anderson, and Narahashi, 1966).

METHODS

The sucrose-gap voltage-clamp method of measuring ionic currents in squid giant axons was essentially the same as previously described by Moore, Narahashi, and Ulbricht (1964). The only significant change was to rearrange the sucrose flow to obtain more reliable insulation between the central and lateral pools and somewhat more convenient control of the shape and size of the artificial "node."

Leakage currents (observed for small positive and negative potential steps from the holding level) were not constant, but decayed exponentially to a final level of about one-half the initial value. Therefore, we estimated the leakage current by the equation $I_L = (I_0 - I_\infty)e^{-t/\tau} + I_\infty$ where the subscripts represent the leakage current at times $t = 0$ and $t = \infty$ (actually a few milliseconds). The time constant, τ, was in the neighborhood of 0.4 ms. The actual values for I_∞/I_0 and τ were chosen from records for hyperpolarizing potential steps for each experiment and the leakage current subtracted from the total current to obtain the peak sodium and steady potassium currents.

The solutions used to bathe the node are given in Table I. The experiments were done at a temperature of 5–10°C. Three to six consistent and reproducible experiments were deemed sufficient for each experimental situation and a representative example was chosen for illustration.

RESULTS

Natural and/or Artificial Sea Water For purposes of comparison and reference, some characteristic effects of TTX on squid axon membrane ionic

TABLE I
COMPOSITIONS OF EXTERNAL SOLUTIONS

Solution	Na	Li	K	Cs	Rb	Ca	Mg	Cl	SO_4	HCO_3
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
Artificial sea water	432.19		9.18			9.46	49.4	504.9	26.0	2.19
Na	526		10			50		636		
Li		526	10			50		636		
K			460			50		560		
Cs			10	526		50		636		
Rb					460	50		560		
½ Na + ½ K	216		216			50		532		

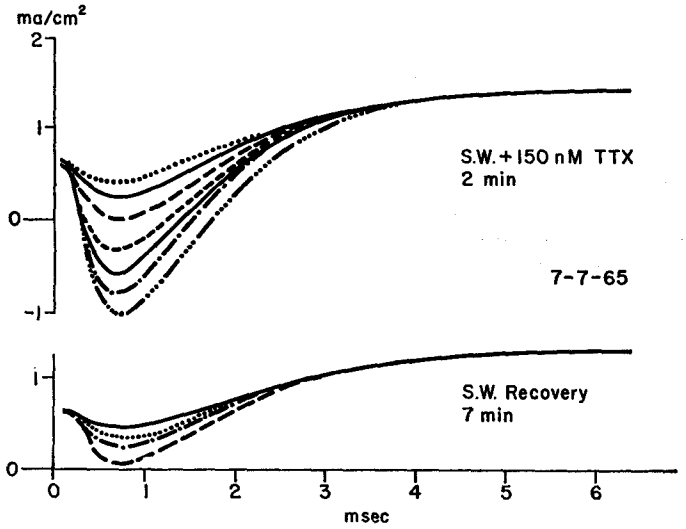

FIGURE 1. Top, selective blockage of inward transient sodium current in voltage-clamped squid axon by tetrodotoxin. The membrane potential was pulsed to a constant potential and the resulting current observed. The lowest curve was the current pattern prior to the addition of 150 nM tetrodotoxin. Successive records were taken at 15 sec intervals over a period of 2 min. The early downward transient (inward current) was reduced while the late steady current was unchanged. The last few curves were almost identical and two have been omitted for clarity. Below, partial recovery of the early transient inward (downward) current over a 7 min washout period.

currents resulting from a voltage-clamp step of membrane potential are shown. Fig. 1 shows the current patterns at a constant potential step observed at 15 sec intervals over a period of 2 min following the addition of 150 nM of TTX to the artificial sea water flowing past the node. While the late steady outward current remains unchanged, the early transient inward Na current is reduced from about 1.5 ma/cm² to nearly zero. Partial recovery of the Na current is seen during a 7 min wash period following the TTX treatment. There is no

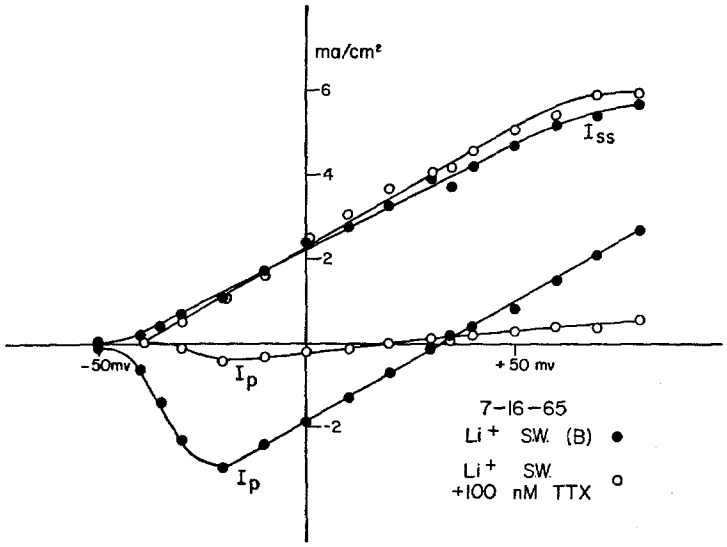

FIGURE 2. Current-voltage relations in an axon membrane bathed in lithium sea water. The branch marked I_{ss} is the late steady outward potassium current. The lower branch is the peak transient inward lithium or outward sodium current. A few minutes after the addition of 100 nM tetrodotoxin to the lithium sea water, the peak transient currents are selectively blocked.

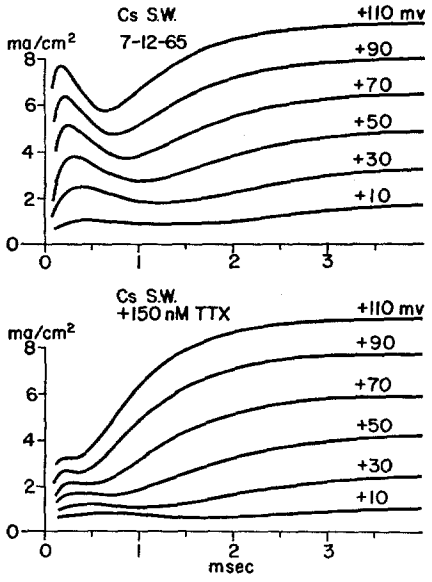

FIGURE 3. Families of current-time curves in an axon membrane bathed in cesium sea water. The absolute value of the voltage-clamped membrane potential is shown at the right of each curve. The lower records were taken 2–3 min after addition of 150 nM tetrodotoxin.

change in the time to the peak transient current throughout the experiment. This illustrates previous observations that there is a reduction of the transient current without a concurrent change in its kinetics (Takata et al., 1966).

Lithium Sea Water It is well known that Li can substitute for Na in the generation of action potentials in axons (Overton, 1902; Hodgkin and Katz, 1949; Narahashi, 1963). Current-voltage relations for a squid axon in lithium sea water are shown in Fig. 2 and are essentially identical to those for natural or Na sea water. The early transient inward current in Fig. 2 is carried by lithium; the early outward transient is primarily carried by sodium. When 100 nM of TTX is added to the lithium sea water, the early transient currents are selectively reduced, lithium inward and sodium outward. The late steady potassium current was not affected in this experimental condition.

Cesium Sea Water It has been shown previously that Cs is a poor substitute for Na in traversing the early transient channel (Pickard, Lettvin, Moore, Takata, Pooler, and Bernstein, 1964; Chandler and Meves, 1965; Moore, Anderson, Blaustein, Takata, Lettvin, Pickard, Bernstein, and Pooler, 1966) and for potassium in the late steady channel opened by depolarization of the membrane (Chandler and Meves, 1965). Thus, in changing from Na to Cs sea water one would expect little or no change in the late steady current carried outwardly by K ions. However, because Cs cannot substitute for the external Na it replaced, the sodium equilibrium potential is shifted by some 75 mv toward a more negative inside potential. Thus, at all potentials there are large outward early transient currents carried primarily by Na as shown in the upper part of Fig. 3.

The effect of TTX at a concentration of 150 nM is shown in the lower half of Fig. 3. It is clear that the early outward sodium current is reduced without effect of the late steady potassium current. These results are replotted as current-voltage relations in Fig. 4. The observed decrease in the sodium conductance may be less marked than usually seen in natural sea water at this TTX

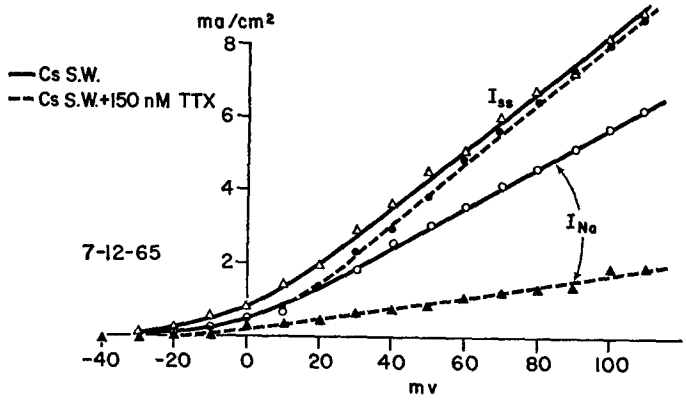

FIGURE 4. Current-voltage relations plotted from Fig. 3. The late steady outward current, I_{ss}, is essentially unaffected by tetrodotoxin in cesium sea water while the early outward transient sodium current is largely blocked.

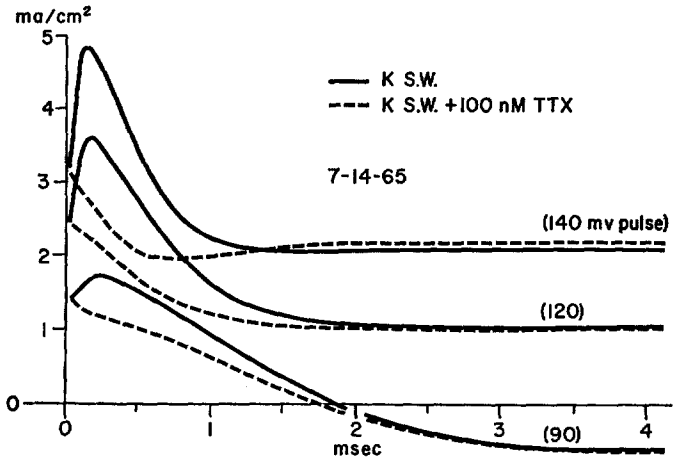

FIGURE 5. Current-time curves in an axon membrane bathed in potassium sea water and a few minutes after the addition of 100 nM tetrodotoxin. In this experiment the base line changed during the course of the experiment and the curves are marked with the applied step of potential change.

concentration but there is no doubt about the fact that the flow of outwardly directed early sodium current is drastically and selectively blocked.

Potassium Sea Water When the axon is bathed in potassium sea water, the internal and external concentrations of potassium are nearly equal resulting in a potassium equilibrium potential near zero. Thus it is possible to have late steady inward potassium currents over a range of negative potentials limited because of the exponentially decreasing conductance in the neighborhood of the normal resting potential (Moore, 1959).

In potassium sea water, there is an early outward transient current through the axon membrane carried primarily by sodium leaving the axoplasm. In Fig. 5 it can be seen that this early outward transient current is blocked by 100 nM of TTX while the late steady potassium current inward or outward is not affected.

Additional records of larger inward potassium currents, taken at slower sweep speeds and more negative potentials, are shown in Fig. 6. Because of a large shift of the zero base line during the experiment on axon 7-14-65 the absolute membrane potential was uncertain. Therefore we have labeled these curves according to the size of the applied step or pulse. It is again evident from the middle section of Fig. 6 that there is no blockade of inward potassium current by addition of TTX to the K sea water.

Rubidium Sea Water Rubidium can substitute for potassium in the external bathing solution (Pickard et al., 1964) and carry late steady inward current although not always as well (Moore et al., 1966). In the lower part

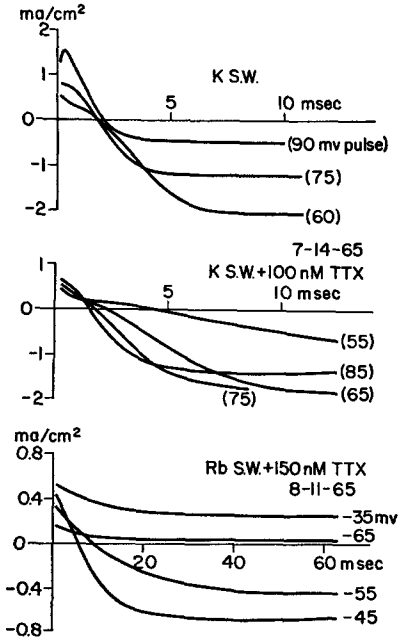

FIGURE 6. Upper and center, current-time curves on same axon with experimental conditions as in Fig. 5 but at slower sweep speeds. Lower, current-time curves for an axon membrane in rubidium sea water containing 150 nM tetrodotoxin.

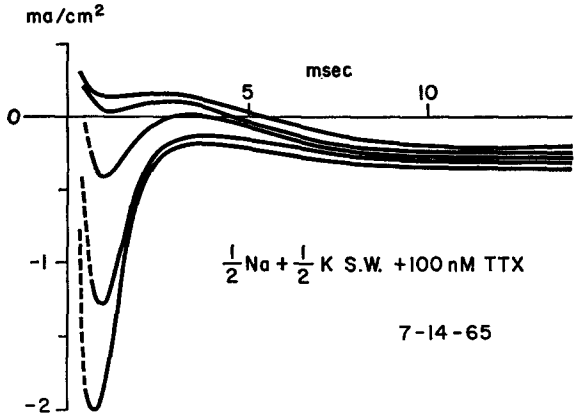

FIGURE 7. Selective effect of tetrodotoxin added to a mixture of sodium and potassium sea waters. The lowest curve is the voltage-clamped axon membrane current before the addition of 100 nM tetrodotoxin. The early transient inward current was rapidly reduced (curves taken at about 30 sec intervals) while the late inward current was only slightly affected.

of Fig. 6 it can be seen that Rb currents can still flow inward in the presence of 150 nM TTX.

One-Half Na + One-Half K Sea Water When using a bathing solution consisting of equal amounts of Na and K, it is possible to find a potential region

in which both the early transient current and the late steady current are inward. Fig. 7 shows the essentially complete blockade of the early transient while the late maintained current is only slightly reduced if at all. We attribute most of this reduction in the late current to a drift in the area of the artificial node because of slight changes in the pattern of solution flow past the nerve. The small early current in the latest and uppermost record occurring at about 0.5 msec is of the shape usually attributed to leakage and is the mirror image of the early current pattern seen upon hyperpolarization.

DISCUSSION

These data on the effects of TTX on monovalent cation currents provide some answers to the questions set forth in the Introduction. (*a*) The fact that TTX blocks the transient influx of Li as well as the transient influx of Na shows that the action of TTX is not based on a selective effect on the Na ion per se. (*b*) The early transient outward current in Cs, K, and Rb sea waters was inhibited by TTX, while late steady-state influx of K and Rb was not affected. Thus the selectivity of TTX cannot be based on a discrimination between the inward and outward flow of positive ions. (*c*) In every experiment the early transient current, regardless of the direction of net flow or the ion carrying this current, was depressed by TTX. The late steady current, on the other hand, was unaffected by TTX, irrespective of the direction of net flow and the ion involved. The results are therefore consistent with a mechanism of TTX action based on its ability to distinguish between an early transient conductance path and a late steady conductance path, and to block only the former.

Support for this hypothesis comes from some recent studies of Chandler and Meves (1965). It has been shown that in axons internally perfused with KCl solutions, when the inside is made strongly positive, there is an early outward transient current, presumably carried by K. This has been interpreted as evidence that the membrane is one-twelfth as permeable to K as it is to Na during the early transient period. Recent experiments by Chandler and Meves (personal communication) show that this early outward current is blocked in the presence of external TTX. This therefore substantiates our observations that TTX does not appear to block the early transient by discriminating against the Na ion per se, but rather that it appears to block any ion carrying the early transient current.

A further test of this hypothesis stems from the data on various amines which may act as sodium substitutes in nerve. Studies on frog nerve (Larramendi, Lorente de Nó, and Vidal, 1956; Lorente de Nó, Vidal, and Larramendi, 1957) and on the perfused, isolated squid axon (Tasaki, Singer, and Watanabe, 1965) demonstrate that action potentials may be elicited when NH_3^+, guanidinium$^+$, or hydrazinium$^+$ ions are added to sodium-free

external media. In the squid axon, the action potentials obtained in the presence of these ions are all effectively blocked by TTX (Tasaki and Singer, 1966; Tasaki, Singer, and Watanabe, 1966). Voltage-clamp studies by Binstock (personal communication) on NH_3^+, and by Chandler and Meves (personal communication) on guanidinium$^+$, show that the early transient current may be carried by these ions, although they are somewhat less effective than Na^+. Furthermore, the early transient current in a guanidinium solution is blocked by TTX (Tasaki, Singer, and Watanabe, personal communication). Taken together, these results seem to further confirm the notion that it is neither the Na nor even the Li ion per se, which is blocked by TTX, but rather any ion which carries the early transient current.

There is, however, a recurring observation by a number of investigators that the apparent sodium equilibrium potential (that potential at which there is neither an inward nor an outward early transient current) reversibly decreases when TTX is applied (Nakamura et al., 1965; Takata et al., 1966). As noted in the Introduction one of us (MPB) in unpublished experiments on lobster axons has observed a change in the early transient "equilibrium potential" from an initial value of +57 mv to +30 mv after 3.5 min in 90 nM TTX; there was an early outward transient current at +57 mv in the presence of the TTX. The shift in the equilibrium potential is especially apparent in experiments in which the external Ca^{++} concentration is high (Takata et al., 1966; MPB, unpublished data).

The notion that TTX may not block the potassium as well as the sodium in the early transient channel may be an explanation for this recurring observation. On the basis of the Chandler and Meves observation of the potassium to sodium permeability ratio of one-twelfth during the early transient, a preferential block of sodium would lead to a decrease in the apparent Na equilibrium potential. In the results section (cesium), it was noted that TTX was possibly somewhat less effective in blocking the early transient outward current (than in blocking the early transient inward current in other experiments). This might be a reflection of such a preferential block of sodium, the residual current being carried by potassium and leading to an apparent shift in E_{Na}. Alternatively, if there was a slight directional difference in the TTX blockage of the flow of sodium, one might expect to see some rectification rather than a shift in the equilibrium potential.

Although our experiments clearly show that 100 to 150 nM TTX blocks the inward early transient movement of Na or Li we cannot be certain about its ability to block early transient K movements as effectively. The shift in E_{Na} may be interpreted as caused by a differential effect on the ions traversing the early channel. However, the Chandler and Meves experiments (with a somewhat higher concentration of TTX) give rather direct evidence that K movement is blocked. Pertinent to this problem, it must be noted that the

precision with which the equilibrium potential can be determined is a function of the relative magnitudes of the early transient and leakage currents. This discrimination becomes particularly difficult when the transient current is drastically reduced, as in the case when TTX is used externally. Another difficulty in the resolution of the early sodium current has been pointed out by Nakamura et al. (1965). If TTX effectively blocks all the transient current (including that fraction carried by K^+), the relative importance of the small fraction of the build-up of the late steady (primarily K^+) current may be magnified and account for the apparent shift in E_{Na}.

On the basis of the data presented in this paper, as well as previous data on the effect of TTX on the voltage-clamped axon, it is tempting to speculate that the early transient and late steady conductance pathways are spatially separated in the nerve membrane. However, it should be clear that the results above do not per se provide any information about the geometry or spatial location of the two channels. Rather, the channels are shown to be operationally different.

The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department or the naval service at large.

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