Blockage of Sodium Conductance Increase in Lobster Giant Axon by Tarichatoxin (Tetrodotoxin)

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ABSTRACT Tarichatoxin, isolated from California newt eggs, has been found to selectively block the increase of sodium conductance associated with excitation in lobster giant axons at nanomolar concentrations. This resulted from a reduction in the amplitude of the conductance increase rather than a change in its temporal characteristics. The normal potassium conductance increase with depolarization is not altered. A high concentration of calcium applied concomitantly with the toxin significantly improves the reversibility of the sodium blocking. This toxin has recently been identified as chemically identical with tetrodotoxin from the puffer fish. Toxins from the two sources are equally effective and are shown to have an action which is distinctly different from that of procaine.

INTRODUCTION

Tarichatoxin is extracted from the eggs of the California newt *Taricha torosa*. Its discovery, purification, pharmacology, and structure have been reviewed recently by Mosher, Fuhrman, Buchwald, and Fischer (1964) in *Science*.

Turner and Fuhrman (1947) demonstrated its blocking action on isolated frog sciatic nerves using a semipurified material. With the recent purification and crystallization of the toxic principle (Brown and Mosher, 1963), the term *tarichatoxin* was proposed and its biological activity reinvestigated by Kao and Fuhrman (1963). They confirmed the earlier observation, finding that blocking of desheathed frog sciatic nerve occurred without any depolarization at extremely low concentrations of the toxin (1 to 10 μg/liter or about 0.003 to 0.03 μM).

Because of this high potency, it was decided to study the mode of action of

this toxin by the voltage-clamp method. Such an investigation should lead to a better understanding of the ionic mechanisms responsible for excitation and show whether its action was similar to or different from that of tetrodotoxin, which also has been found to be extremely potent. At the time, the provisional empirical formula for tarichatoxin was $C_{11}H_{17}N_3O_8$ (Brown, 1962) while that for tetrodotoxin appeared to be different; Tsuda and Kawamura (1952) favored $C_{12}H_{19}N_3O_9$ for tetrodotoxin but Yokoo (1950) preferred $C_{12}H_{17}N_3O_{10}$.

In a preliminary study on the effect of tetrodotoxin on lobster axons, Narahashi, Moore, and Scott (1964) had found that the sodium conductance increase was selectively blocked. The present experiments on tarichatoxin were begun subsequently with a view toward a comparison of the actions of the two toxins. When the present experiments were nearly completed, evidence became available that the two toxins were identical (Buchwald et al., 1964; and Mosher et al., 1964). It is now generally agreed[1] that the formula for the toxins is that originally proposed by Brown (1962); *i.e.*, $C_{11}H_{17}N_3O_8 \cdot 1/2H_2O$.

While the present experiments confirm the findings on tetrodotoxin (Narahashi et al., 1964), the investigations are much more extensive than the previous one, and the blockage can now be distinguished as quite different from that brought about by local anesthetics.

METHODS

Voltage-clamp measurements were performed on giant axons in the circumesophageal connectives of the lobster *Homarus americanus* by means of the sucrose-gap technique in essentially the same way as described by Julian, Moore, and Goldman (1962 *a*, *b*). Two streams of sucrose separated the axon's surface into three segments in contact with streams of electrolyte. The very short central portion or "artificial node" (50 μ or less) was bathed in artificial sea water to which tarichatoxin was added for the experimental solution. The membrane potential of this node was measured as a potential between the central pool and a lateral pool containing one end of the axon in KCl. Stimulating and/or clamping currents were injected into the other lateral pool containing the other end of the axon also in KCl. The "holding potential" or steady value of potential to which the voltage clamp pulses were added was maintained between -80 and -100 mv throughout the experiment in order to assure complete removal of any inactivation of the sodium-carrying system.

The compositions of the artificial sea water flowing past the central node, the sucrose solution, and the high potassium solution in the lateral pools were the same as used previously (Narahashi et al., 1964). The rate of flow of the solutions was relatively high so that changes in solutions applied to the node could be made rapidly. The time for a 95% complete change in the solution surrounding the node was estimated at 15 sec from other experiments in which the time course of the membrane potential change was followed after a change in the external potassium concentration.

[1] Tsuda, K., Ikuma, S., Kawamura, M., Tachikawa, R., Sakai, K., Tamura, C., and Amakasu, O., 1964, *Chem. Pharm. Bull. Tokyo*, **12**, 1357.

A stock solution of 500 μg/ml of crystalline tarichatoxin was made up in distilled water made slightly acidic with acetic acid. From this, further dilutions to 0.005 to 0.05 μg/ml were made in artificial sea water. Although the stock solution and dilutions were made up on the weight per volume system, the molecular weight now appears well enough established at 330 so that the concentrations will hereafter be expressed in nanomoles/liter (10^{-9} moles/liter = nM). The experiments were conducted at a temperature of about 7°C.

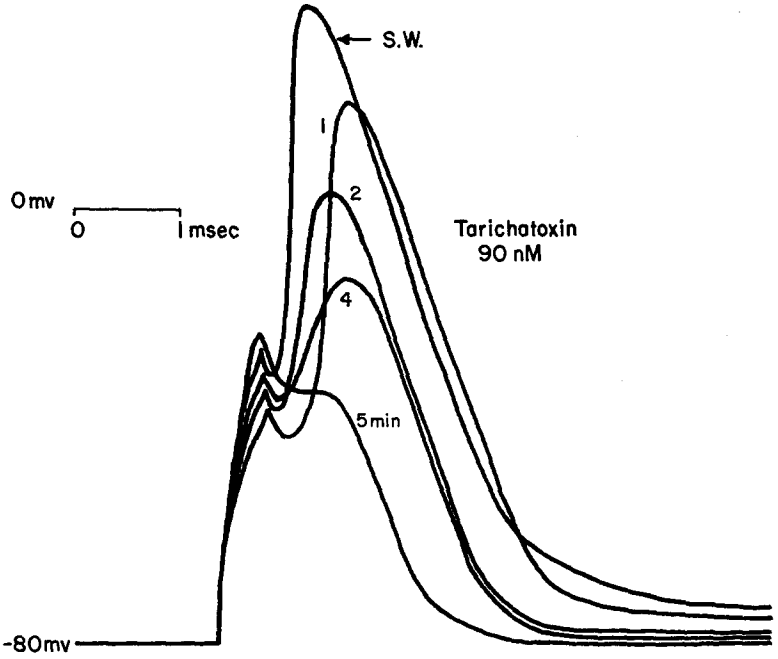

FIGURE 1. Action potential blocking by 90 nM of tarichatoxin; the period of application is shown adjacent to the attenuated spikes.

RESULTS

1. *Action Potentials*

As originally observed by Julian et al. (1962 *a*), there was a marked hyperpolarization of the node upon starting the sucrose flow. This high "resting potential" (80 to 120 mv inside negative) resulted in action potentials in unclamped membranes as high as 160 mv in amplitude. Fig. 1 shows the effect of tarichatoxin at a concentration of 90 nM on the shape of the action potential as a function of the duration of treatment. The base line potential was not affected but the action potential was blocked within about 5 min. Partial blockage of the action potential was usually seen within the same length of time at concentrations as low as 15 to 30 nM. Recovery after washing with sea water was quite variable. If the node was returned to sea water after only par-

tial blockade, such as indicated by the 2 min curve in Fig. 1, sometimes almost complete recovery could be obtained. However, if the action of the toxin were allowed to continue, there was little or no recovery for an axon in the condition of the 5 min curve of Fig. 1 when returned to sea water.

2. *Voltage-Clamp Currents*

The currents observed in voltage-clamped conditions are summarized in the usual manner in Fig. 2. It is apparent that tarichatoxin selectively affects the early transient inward current phase which has been identified as one of sodium ionic current. In contrast the late outward steady-state potassium cur-

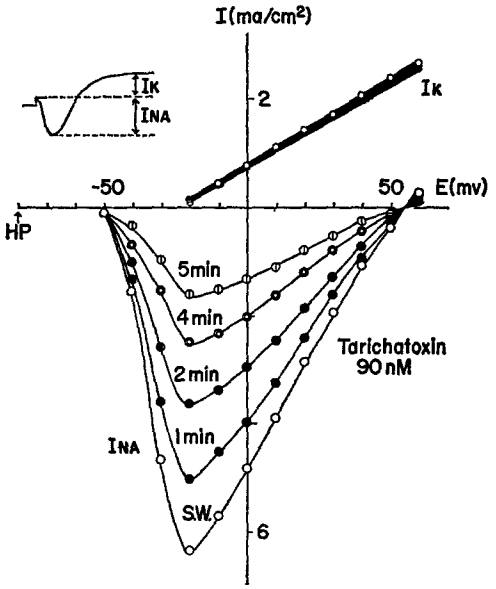

FIGURE 2. Sodium and potassium current-voltage relations observed in the voltage clamp at several intervals following application of 90 nM of tarichatoxin. Same axon as in Fig. 1.

rent is not affected. Fig. 2 also shows that the action of the toxin was to continue to depress the sodium current as long as it was applied in this experiment. Although recovery of the ability to carry a large sodium current was not very marked after this strong depression, complete reversibility was often observed in experiments in which a lower concentration of toxin was applied for a shorter period of time to produce a depression in the sodium current of not more than 50%. No attempt was made to correlate the spike height or maximum rate of rise with the sodium conductances in the toxin-treated axons because the depression of the sodium conductance was changing so rapidly.

Fig. 3 *a* shows the time course of the reduction of the sodium currents brought about by a tenfold range of tarichatoxin concentrations. The sodium current is almost completely blocked in a few minutes by concentrations of 120 nM or greater. Concentrations lower than 15 nM were not used because the

longtime stability of the preparation was not good enough to distinguish toxin effect from normal deterioration of the sodium current with time. A different area of the same axon was used for each toxin concentration shown.

In order to see the relation between the toxin concentration and the depression of the sodium current, data from three to seven axons were averaged for each concentration. The maximum sodium current (relative to the initial sea water value) is shown as a function of the toxin concentration for three in-

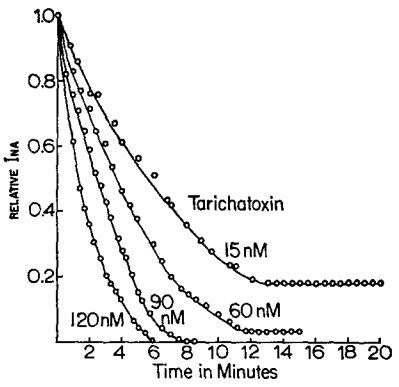

FIGURE 3 *a*

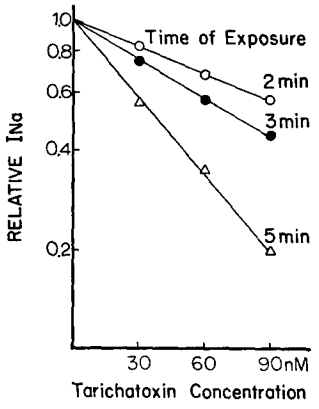

FIGURE 3 *b*

FIGURE 3 *a*. Time course of the reduction of sodium current at a constant potential as a function of tarichatoxin concentration. The ordinate gives the magnitude of the I_{Na} in toxin relative to that in sea water.

FIGURE 3 *b*. Reduction of sodium current at a constant potential as a function of time for various tarichatoxin concentrations.

tervals of toxin application in Fig. 3 *b*. The logarithm of the relative sodium current was chosen for the ordinate because this gave reasonably good straight lines. We do not feel that this relation should be emphasized because of the small range of concentrations and the limited number of experiments used.

The sodium current depends on a number of factors. It is necessary to raise and answer the question as to whether one or multiple factors were changed by tarichatoxin. We therefore compared the sodium inactivation and the time for the sodium current to reach half-maximum in normal and toxic sea waters to see whether or not the factors m and h of the Hodgkin-Huxley formulation had been changed (m and h are the factors governing the transient turning on and off of the sodium conductance).

3. *Inactivation*

The steady-state inactivation of the sodium conductance, called $1\text{-}h_\infty$ in the Hodgkin-Huxley (1952 *c*) formulation, and the time constant for the removal

of inactivation, τ_h, were both measured in the classical way (Hodgkin and Huxley, 1952 *b*). Fig. 4 shows the relative sodium current available during a standard test pulse as a function of the potential applied for some 20 msec just preceding the test pulse. Because the sodium current available at any given test potential was being rapidly reduced (Fig. 3 *a*), it was not possible to obtain a really independent measure of h_∞. However, it was usually possible to take the required data within less than 30 sec so that the net change in the sodium current (without a pulse preceding the test one) was usually less than 10% of its sea water value. Under these circumstances, there was no significant shift in the steady-state inactivation as illustrated in Fig. 4. The time constant of the process of inactivation removal (τ_h) was studied by the classical Hodgkin-Huxley method of applying two potential pulses to the membranes.

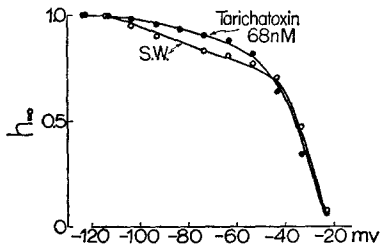

FIGURE 4. Curves for the steady-state sodium inactivation in an axon in sea water and treated with 68 nM of tarichatoxin. The ordinate gives the relative peak sodium current available at a constant potential during a pulse after the membrane had been held at the potential shown for 20 msec or more. The normalizing current in sea water was 7 ma/cm² and 5.3 in the toxin.

The first was chosen to have the sodium channel fairly well inactivated at the end of the pulse. The recovery from inactivation was measured as the relative amount of the sodium current in the second pulse as a function of the time interval between the cessation of the first and the initiation of the second; the resulting normalized current curves are shown in Fig. 5. Although the small differences between the curves in sea water and in toxin for both Figs. 4 and Fig. 5 border on the range of experimental variations or approach the resolution of the method in this type of study, they appeared to occur consistently; we have no explanation for the small differences. We conclude that inactivation, $1 - h$, in the Hodgkin-Huxley equations is not appreciably changed by the application of toxin. With tarichatoxin there may have been a small reduction in the time constant for recovery from inactivation of the sodium channel by repolarization. However, this variable actually has no influence on the usual type of voltage-clamp current measurements shown in Fig. 2 because there the membrane was repolarized for at least a full second between test pulses.

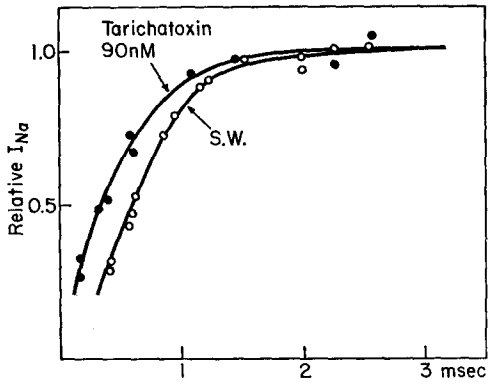

FIGURE 5. Plots of the inactivation removal, τ_h, for an axon in sea water and treated with 90 nM of tarichatoxin studied by the two pulse method. The normalizing currents in the first pulse were 7 and 2 ma/cm² respectively.

4. *Sodium Current Onset*

In order to see whether tarichatoxin causes a change in the factor m which affects the way in which sodium current is turned on following a step change in potential in the Hodgkin-Huxley equations, the time between the onset of the potential pulse and the time to one-half the peak of the inward current, $t_{p/2}$, was measured and plotted as a function of membrane potential. The curves for a typical experiment are shown in Fig. 6. There does not appear to be any appreciable difference between the values of $t_{p/2}$ in sea water and in the toxin and we may conclude that the variable m is not changed by tarichatoxin.

A further test for the constancy of the fast time course of the sodium conductance system was to see whether the toxin-reduced current could be multiplied by a scale factor such as to make it match with the normal sea water current. When such a constant multiplying factor (obtained from the ratio of the peak currents) was used, the current patterns did, in fact, coincide. This was taken as further evidence that neither the m nor the h parameter had changed, at least in the potential range eliciting large inward currents.

5. *Calcium Protection*

A number of studies were performed in which the concentration of the calcium in the toxin-containing sea water was varied over a considerable range (at the expense of sodium) to see whether there was any change in the sodium-depressive action of the toxin. For purposes of comparison, Fig. 7 (left) gives a representative experiment in which the calcium concentration was maintained at the normal level of 25 mM throughout. Typically, this drastic depression of the sodium conductance by the toxin is only slightly reversed after washing. If the calcium concentration is increased fourfold during the toxin application (Fig. 7, right), there is nearly the same reduction in the sodium conductance but the most striking effect is on the reversibility of the sodium

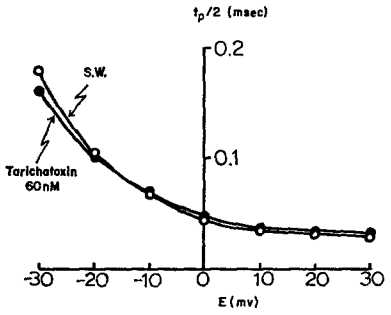

FIGURE 6. Comparison of the time for the sodium current to reach one-half of its peak value in sea water and in 60 nM of tarichatoxin when the magnitude of the peak current was reduced by 40%.

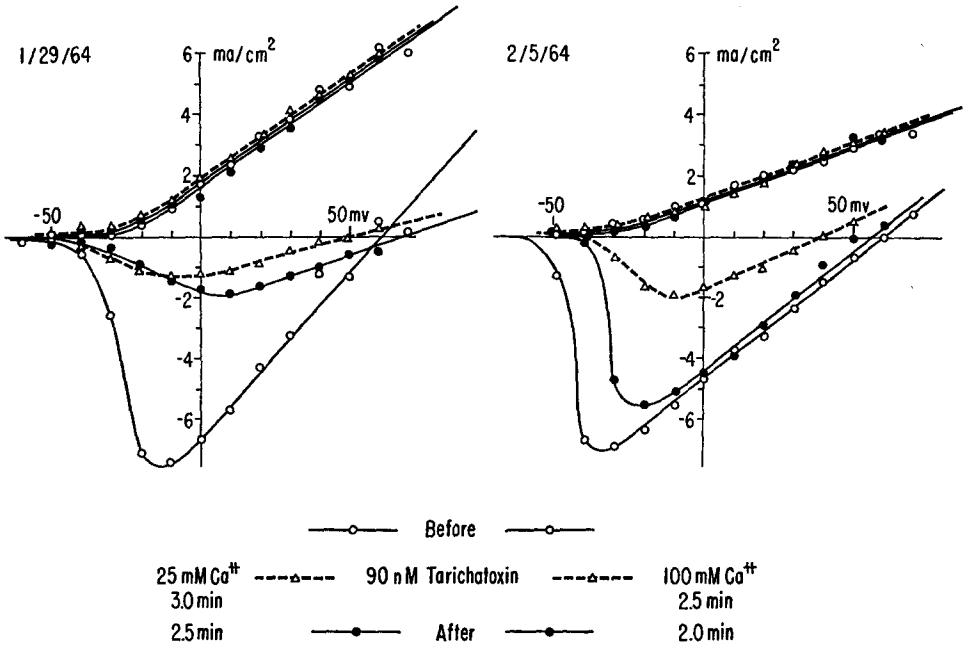

FIGURE 7. Current-voltage relations for axons treated with 90 mM tarichatoxin. Left, calcium concentration maintained at 25 mM throughout. Right, calcium concentration raised to 4 times normal during toxin application. Because of apparent nodal area changes, scale factor corrections were applied to the observed currents during recovery phase left, 1.3, right, 0.73.

conductance. There were potassium conductance changes for the recovery phases of these two experiments which were thought to represent unobserved changes in the "nodal" area because in nearly all other cases no potassium conductance change was seen. Therefore, the observed recovery phase currents were scaled to match the previous values. These corrections are in a direction to minimize rather than to enhance the differences. Therefore it seems clear that the recovery of the ability to pass high sodium currents is much more marked in the axon in which high calcium was applied along with

the toxin. In contrast, in other experiments in which the calcium was reduced to one-tenth of its normal value during the toxin application, there was little or no recovery of the sodium conductance mechanism.

DISCUSSION

The present observations that tarichatoxin selectively blocks the normally occurring increase of sodium conductance upon depolarization are essentially the same as those found for tetrodotoxin (Narahashi et al., 1964). Indeed, the effective concentrations and the time course of the blockade of the sodium current were also very close even though the experiments were performed with the two toxins obtained from entirely different sources. The present data, using tarichatoxin, are much more extensive than those obtained with tetrodotoxin, but in view of the identity of the two toxins (Buchwald et al., 1964) the present results and conclusions can now be applied to tetrodotoxin as well.

There are several distinctive differences in the manner of blockage of nerve excitation between local anesthetics and tarichatoxin (or tetrodotoxin, dubbed TTX by K. S. Cole). Because procaine is the only other agent studied in as much detail, we will limit our comparison to these two.

A. *Concentration Ratio for Blocking* Procaine reduces the ionic conductance of squid and lobster axon membranes at a concentration of about 3 to 4 mM (Taylor, 1959; Blaustein and Goldman, 1965) and blockage is usually sure with a concentration of 10 mM. In contrast, a concentration of only 90 nM of TTX blocks within 5 min (see Fig. 1). The toxin is therefore more effective by a factor of 10^5 in terms of the concentration required.

B. *Selectivity* The toxins have been found to block only the sodium entry, leaving the potassium current unaffected (the present results; Narahashi et al., 1964; Nakamura et al., 1965; Moore, 1965). On the other hand procaine affects the magnitude of both ionic conductances (Taylor, 1959; Blaustein and Goldman, 1965).

C. *Time Course of the Conductance Changes* Our results show that the toxins do not alter the kinetics of the sodium or potassium current increase. Taylor (1959), Blaustein and Goldman (1965), and unpublished experiments in our laboratory have shown that procaine causes a distinct increase in the time for the sodium current to reach its peak. There is also a very marked slowing in the rise of the late potassium current (Taylor, 1959; unpublished experiments in our laboratory).

D. *Location of Action* Procaine appears to be effective in blocking nerve excitation when internally perfused in the squid axon at a concentration which blocks externally (10 mM, Narahashi, personal communication). In contrast, TTX has been found to be ineffective when internally perfused at high con-

centrations (1000 nM) for long times (20 min). Preliminary evidence that TTX was ineffective on the inside of the membrane was shown by Moore (1965); the more definitive results cited were obtained by Narahashi, Anderson and Moore but are not yet published.

E. *Interaction with Calcium* Blaustein and Goldman (1965) report that procaine appears to act at the same site that calcium does. Both cause shifts of the conductance curve along the voltage axis and alter the time course of the conductance changes (see section C). Although our experiments with tarichatoxin were not designed to study this point, it is clear that high calcium gives some protection against the toxin and definitely enhances the ability of the nerve to recover from a strong toxin depression of conductance.

It is also interesting to note that there appears to be a rather permanent shift in the potential at which the sodium current increases rapidly after treatment with toxin and high calcium (see Fig. 7). In the Blaustein and Goldman (1965) experiments with procaine and high calcium, the shift which occurred in the sodium conductance curve was reversible.

The present results agree with and extend earlier reports on the mode of action of tetrodotoxin. Similar selective blockage of the voltage-sensitive sodium conductance systems has been observed in (*a*) the lobster axon by Narahashi et al. (1964), (*b*) the squid axon by Moore (1965) and Nakamura, Nakajima, and Grundfest (1965), and (*c*) the eel electroplax also by the latter authors (1964). The mode of excitation block in frog nerve fibers observed by classical methods (Turner and Fuhrman (1947); Dettbarn, Higman, Rosenberg, and Nachmansohn (1960); and Kao and Fuhrman (1963)) seems to be identical with that reported here from preliminary voltage-clamp studies on the single node by Hille (1966).

Evidence suggesting that the sodium and potassium currents which flow during activity do so through separate pathways appears to be mounting. As a consequence of their finding that cocaine blocked both potassium and sodium channels, Shanes, Freygang, Grundfest, and Amatniek (1959) suggested that the conductance changes were not as distinctly different as might be expected from their time course differences. However, the selectivity of the toxin block of the channel, placed beside the selective block of the potassium current by internally injected tetraethyl ammonium chloride (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965) certainly speaks strongly for different pathways for these two ions.

One cannot say what the mechanism of the action of the toxin is until the mechanism of the normal sodium and potassium conductance changes is understood. However, it does appear that the toxin acts in a very specific and effective manner to inhibit the normal voltage-sensitive sodium conductance increase in nerves. In frog skeletal muscle in which sodium conductance also appears to increase upon depolarization, Narahashi et al. (1960) found that it

blocked excitation, leaving the steady rectification system intact. On the other hand, it does not block the acetylcholine-induced sodium conductance increase in the end plate region of the neuromuscular junction (Furukawa, Sasaoka, and Hosoya, 1959). Nor does it affect the voltage-sensitive calcium conductance increase in the barnacle muscle (Hagiwara and Nakajima, 1965). Because saxitoxin (from mussels) blocks nerve activity at concentrations near that for tetrodotoxin and has a guanidinium group on one end of the molecule as does the latter, it has been suggested (Kao and Nishiyama, 1965) that the guanidinium enters the sodium pathway and is held there by reactions between the other parts of the molecule and the cell membrane surface.

The very high specificity of the block of sodium ion pathways which are voltage-sensitive and the very low concentrations of the toxin required encourage us to look forward to the use of derivatives of the toxin. Studies with them should be most useful in characterizing the molecular architecture and properties of the membrane responsible for excitation and perhaps lead to the development of a new class of local anesthetics.

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