Selective Modification of Sodium Channel Gating in Lobster Axons by 2, 4, 6-Trinitrophenol

Evidence for Two Inactivation Mechanisms

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Abstract Trinitrophenol (TNP) selectively alters the sodium conductance system of lobster giant axons as measured in current clamp and voltage clamp experiments using the double sucrose gap technique. TNP has no measurable effect on potassium currents but reversibly prolongs the time-course of sodium currents during maintained depolarizations over the full voltage range of observable currents. Action potential durations are increased also. $\tau_m$ of the Hodgkin-Huxley model is not markedly altered during activation of the sodium conductance but is prolonged during removal of activation by repolarization, as observed in sodium tail experiments. The sodium inactivation versus voltage curve is shifted in the hyperpolarizing direction as is the inactivation time constant curve, measured with conditioning voltage steps. This shift speeds the kinetics of inactivation over part of the same voltage range in which sodium currents are prolonged, a contradiction incompatible with the Hodgkin-Huxley model. These results are interpreted as support for a hypothesis of two inactivation processes, one proceeding directly from the resting state and the other coupled to the active state of sodium conductance.

Control of the movement of ions through the sodium and potassium channels in nerve membranes in response to changes in the membrane electric field is commonly referred to as gating. In the Hodgkin-Huxley (H-H) model of membrane excitation (Hodgkin and Huxley, 1952 b) the gating properties of the sodium channel are represented by two independent factors. An activation factor, $m$, describes the opening and closing of channels upon depolarization and repolarization, while an inactivation factor, $h$, governs the slower decline in sodium conductance when depolarization is maintained.

Experimentally it has been possible to segregate the inactivation and activation processes on the basis of their differential sensitivities to certain modi-
fication procedures. Sodium inactivation can be altered or even removed through the use of enzymes (Armstrong et al., 1973), iodate ions (Stämpfli, 1974), photodynamic techniques (Pooler, 1972), and certain marine toxins and scorpion venoms (Narahashi et al., 1969; Narahashi et al., 1972; Koppenhöffer and Schmidt, 1968; Cahalan, 1975). Recently a reasonably selective modification of sodium activation was achieved using a Centruroides venom (Cahalan, 1975).

The formal independence of the m and h factors of the H-H model has been questioned on both theoretical and experimental grounds (Hoyt, 1968; Hoyt and Adelman, 1970; and Goldman and Schauf, 1972), which has stimulated a search for new models incorporating activation-inactivation coupling (e.g. Goldman, 1975; Moore and Cox, 1975). Also discrepancies have been observed between values for the sodium inactivation time constant obtained during a depolarizing test pulse on the one hand, and from the effects of conditioning prepulses on the other hand (Goldman and Schauf, 1973), raising questions about the nature of the molecular event(s) controlling sodium channel inactivation.

In this paper we report on a selective alteration of sodium channel gating in the lobster giant axon membrane by 2,4,6-trinitrophenol. The results of this investigation provide experimental evidence for two inactivation processes governing the decay rate of $g_{Na}$, one being independent of the m process and the other being coupled directly to it. The reported results are consistent with a previous preliminary study of TNP on lobster axons (Cooke et al., 1968).

METHODS

Experiments were performed on single giant axons isolated from the circumesophageal connective of Homarus americanus using a double sucrose gap technique. Details of basic nerve chamber design and electronic apparatus for control of membrane current and voltage have been described elsewhere (Julian et al., 1962 a, b; Pooler, 1968). The artificial seawater (ASW) bathing the experimental patch of membrane in the central pool contained ions in the following concentrations (mM): Na+428, K+10, Ca++50, Mg++8, Cl-546, SO4-4, and was buffered to pH 7.8 (at 2°C) with 1 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). The isosmotic sucrose solution (725 mM) which borders the artificial “node” in the central pool was doped with 10^-4 M CaCl₂ resulting in isolation resistances measured in the absence of an axon of 10-15 MΩ between adjacent ASW pools. These values fall within a normal acceptable range observed previously using only deionized sucrose (see Moore, 1971). This Ca++ doping procedure significantly enhanced the stability of a given nodal area and greatly extended the number of viable areas obtained per axon without compromising the measurement of potentials. Details of the procedure will be presented in a later publication.

Experimental solutions of TNP (1 mM) were prepared by dissolving 2,4,6-trinitrophenol (Sigma Chemical Co., St. Louis, Mo.) in ASW and adjusting the pH to
control level with NaOH. The central pool input could be switched from ASW to anyone of a number of test solutions with a washout time of approximately 40 s for a complete solution change. The central pool temperature was maintained at 2-4 °C during all experiments.

Data Analysis and Terminology
Oscilloscope traces of membrane current and voltage were photographed on 35-mm film. Enlarged images of the traces were digitized and analyzed using the Hewlett Packard digitizer-calculator-digital plotter system (models 9864A, 9810, and 9862A Hewlett-Packard Co., Avondale, Pa.). Sodium currents were obtained from records of total current by subtraction in time of raw current records before and after treatment with $10^{-6} \text{ M}$ tetrodotoxin (TTX). Time constants $\tau_i$ and $\tau_h$ of the H-H system were obtained from semilogarithmic plots of sodium current versus time, using the method described by Hille (1967). The time constant for inactivation assayed during the falling phase of sodium current $\tau_i$, is operationally distinguished from the time constant assayed by variable duration prepulses which reduce the obtainable peak sodium current during a subsequent test pulse. The latter time constant, referred to here as $\tau_c$, after Goldman and Schauf, (1973), was measured by the method of Hodgkin and Huxley (1952 a). Briefly, values of $\tau_c$ were obtained by plotting peak sodium currents during a standard test pulse (on the positive limb of the sodium $I-V$ relation) against the duration of a depolarizing prepulse and subjecting the obtained points to an asymptotic regression analysis (Snedecor and Cochran, 1967). This inactivation brought about by conditioning prepulses is referred to as conditioned inactivation. A third method for quantifying inactivation kinetics is an inactivation recovery experiment, where twin depolarizing pulses are separated by variable duration repolarizations. The first depolarization inactivates the sodium system, the variable duration repolarization allows varying amounts of recovery from inactivation, and the second depolarization assesses how much recovery has occurred. A plot of peak sodium current during the second depolarization against repolarization duration is also subjected to an asymptotic regression analysis, to yield a time constant for inactivation recovery also referred to as $\tau_c$.

RESULTS
Sodium Currents are Prolonged by TNP
Exposure of the membrane to 1 mM TNP reversibly prolongs the falling phase of sodium currents at all potentials at which currents are observable. This prolongation is shown in Fig. 1 where families of sodium currents from one axon area are shown with and without TNP in the external bath. The prolongation of sodium current results in a reversibly increased action potential duration, as shown in Fig. 2 a. The reversibility of the TNP action is demonstrated in Fig. 2 b, which shows three superimposed membrane currents associated with a clamp step to 0 mV: before TNP, during exposure, and after washout.

The kinetics of TNP action are rather fast as seen in voltage clamp ex-
FIGURE 1. Families of sodium currents from a single membrane area before (upper) and during (lower) exposure to TNP. The associated voltage steps are every 10 mV from -40 to +90 mV. Holding potential = -100 mV.

FIGURE 2. (a) Electrically stimulated action potentials recorded at 1-s intervals during the run-in of TNP. The resting potential is not altered by TNP, but the duration and overshoot are both increased. (b) Reversibility of TNP effect. Ionic currents after a step depolarization to 0 mV from a holding potential of -100 mV. The membrane was held in clamp during exposure and washout of TNP and step depolarized in each case.

Experiments with an 8/s repetition rate where areas of membrane under sucrose are quickly pulled into the nerve chamber central pool containing TNP. The membrane recovers from the sodium-free sucrose and develops full sodium currents within 1 s. The currents which develop are already prolonged and exhibit no delay in TNP action (in contrast to similar experiments with TTX where a considerable delay exists before the TTX effect is observed). The fast action is seen even when many areas are quick-pulled in succession, with many millimeters of axon moved within a 30-s period. Recovery from TNP is much slower, with currents taking several minutes to return to baseline levels.
TNP is Specific for Sodium Channels

No TNP-induced alteration of currents other than sodium has been observed. This is demonstrated in two ways. Fig. 3a shows membrane currents during a step to $-10 \text{ mV}$ in ASW, TNP, and after block of sodium current with TTX. The extra inward current due to TNP is absent after exposure to TTX and is therefore presumed to pass through sodium channels. Absence of any TNP alterations of potassium channels is demonstrated in Fig. 3b. Three families of clamp currents during TTX block of sodium channels are superimposed: showing the results before TNP, after 40-s exposure, and after 2-min exposure. The virtual identity of the current families before and during

\[ \text{Figure 3.} \ (a) \text{ Abolition by TTX of prolonged inward current induced by TNP. The membrane was step depolarized to } -10 \text{ mV in each case.} \ (b) \text{ Families of membrane currents in } 10^{-8} \text{ M TTX showing absence of TNP effect on potassium current. Superimposed sweeps of currents associated with step depolarizations from } -20 \text{ to } +60 \text{ mV were taken before TNP and after exposure to TNP for 40 s and 2 min. The three families were then photographed and are seen to be indistinguishable. The grid divisions represent } 0.14 \mu \text{A and 2 ms.} \]

TNP exposure demonstrates the lack of TNP alteration of the magnitude or kinetics of potassium currents.

Fig. 4 shows peak sodium current versus voltage curves for an area before and during TNP exposure. Small increases in peak current are evident in these data and in the majority of the other experiments performed (see e.g. Fig. 1). The stability of the sodium reversal potential suggests that TNP does not alter channel selectivity, while the absence of a shift along the voltage axis implies that no dramatic change in sodium channel activation has occurred.

Alterations of m and h Kinetics are Complicated

The quantitative aspects of the slowing of inactivation are complex and somewhat variable from experiment to experiment. For test potentials more negative than $-15 \text{ mV}$ the falling phase of sodium current generally followed
Figure 4. Peak sodium current versus voltage relation for a membrane area in ASW (closed symbols) and then in TNP (open symbols).

a single, though slower than normal, exponential time-course in TNP. At increasingly more positive test potentials a second slower component developed which in some experiments clearly remained at a nonzero level even for test pulse durations of 90 ms. A complete description of this complex time-course and its voltage dependence will require further investigation.

Despite the complicated time-course of sodium currents we have measured apparent \( \tau_m \) and \( \tau_h \) values of the H-H system, using the fastest (and earliest) region of the falling phase to define \( \tau_h \). For potentials up to 0 mV at least, the existence of slower components of inactivation should not grossly distort the measurement. The results of this procedure are plotted in Fig. 5a and b. Under the action of TNP \( \tau_h \) values are considerably increased at all potentials, while measured \( \tau_m \) values are not markedly altered. The removal of activation, however, is slowed also, as revealed in sodium tail experiments of the kind illustrated in Fig. 6. This shows superimposed currents for an area consecutively bathed in ASW, TNP, and TTX and subjected in each case to the voltage schedule diagrammed in the inset. The rather fast tail seen in ASW is greatly prolonged by TNP, but decays back to the same level as in ASW. After application of TTX there is only a capacitive-leakage transient.

The prolongation of both sodium tail currents and the normal falling phase of \( I_{Na} \) without concomitant slowing of the rise of \( g_{Na} \) during a test depolarization indicates that TNP selectively retards the decay of activated sodium conductance rather than affecting \( m \) or \( h \) processes per se. This resembles a similar selective alteration of sodium conductance kinetics produced by DDT (Hille, 1968).

**TNP and Conditioned Inactivation**

The sodium conductance mechanism in the axon membrane is subject to inactivation by a small predepolarization which fails to fully activate sodium
FIGURE 5. (a) Sodium activation time constant, $\tau_a$, and (b) the inactivation time constant, $\tau_h$, as a function of membrane potential. In each case measurements on a given membrane area were performed in ASW (solid symbols) and then in TNP (open symbols). Symbols represent mean $\pm$ SEM values where, from negative to positive potentials $n$ was 2, 2, 3, 5, 7, 5, 8, 4, 5, 3, 5, 2, and 4 for $\tau_a$ and 2, 2, 3, 5, 8, 5, 8, 5, 8, 4, 6, 2, and 4 for $\tau_h$ measurements. A smooth curve was drawn by eye through the $\tau_a$ data while lines connect the mean $\tau_h$ values in b.

FIGURE 6. Prolongation of sodium tail current by TNP. The membrane was held in clamp during exposure to ASW, TNP, and then TTX. Currents associated with the voltage schedule shown in the inset were superimposed and photographed. The peak tail current is increased by TNP and the time-course is greatly prolonged, indicating interference with the normally fast removal of activation. The grid divisions are 0.2 $\mu$A and 0.5 ms.

conductance as well as by a maintained larger depolarization. Such conditioned inactivation limits the degree to which the sodium conductance will respond to a subsequent test depolarization. In the H-H model of excitation no distinction is made between conditioned inactivation and inactivation measured as the decay of activated conductance. They are formally equivalent over the entire range of experimental membrane potentials. The voltage and time dependence of conditioned inactivation can be determined
by observing sodium current during a test pulse after changes in the magnitude and duration of conditioning prepulse voltages.

The dependence of steady-state conditioned inactivation \( h_\infty \) on voltage over the range \(-100\) to \(0\) mV is illustrated in Fig. 7 before and during application of TNP. The data points represent the ratio of \( I_{Na} \) after a 50-ms prepulse to \( I_{Na} \) in the absence of a prepulse, as a function of prepulse potential. The smooth curves were fit to the data points according to Eq. 1 of Hodgkin and Huxley (1952 a).

TNP shifts the inactivation curve in the direction of hyperpolarization at \( h_\infty = 0.5 \) by an average of 13.7 mV \((V_{0.5} \pm \text{SEM} = -45.9 \pm 1.9 \text{ mV in ASW, and } -59.6 \pm 2.2 \text{ mV in TNP, } n = 5)\). The steepness of the \( h_\infty \) curve is also decreased slightly by TNP as slope factor values \( (\text{see Eq. 1, Hodgkin and Huxley, 1952 a}) \) rose from \(5.0 \pm 0.22 \text{ mV in ASW} \) to \(6.6 \pm 0.17 \text{ mV in TNP (n = 5)}\).

TNP did not appear to raise the "foot" of the inactivation curve in the potential range investigated here indicating that inactivation was complete for long depolarizations. In other experiments using long-duration test pulses at positive potentials the sodium current did not always decline to a zero level, indicating incomplete inactivation. As a result the inactivation curve may be complex in this potential range.

The time-course of conditioned inactivation at several prepulse voltages is illustrated in Fig. 8 for a membrane area in ASW (upper curves) and an adjacent area during exposure to TNP, (lower curves). Normalized sodium current is plotted against prepulse duration in each case for conditioning voltages of \(-50\) to \(-10\) mV. The solid lines represent asymptotic regression fits to the data points. By comparing the time-courses of conditioned inactivation in the two solutions at a given potential a clear increase in the rate of decline of \( I_{Na} \) is observed upon application of TNP to the membrane. The time constants associated with the conditioned inactivation \( (\tau_c) \) are plotted for several experiments in Fig. 9 b as a function of membrane potential. The mean \( \tau_c \) values measured at potentials more negative than \(-15\) mV are significantly smaller in TNP. As the conditioning voltage becomes more positive, however, the change in kinetics due to TNP reverses direction, the \( \tau_c \) values becoming greater in TNP above approximately \(-15\) mV.

The decrease in \( \tau_c \) for potentials more negative than \(-15\) mV is in obvious contrast to the \textit{increases} in \( \tau_h \) measured from the normal decay of \( I_{Na} \) during a test pulse over much of the same voltage range (Fig. 5 b). This bidirectionality of effect implies that the molecular processes underlying the two kinetic phenomena are different and is clearly inconsistent with the formal equivalence of \( \tau_h \) and \( \tau_c \) in the H-H model.

Another measure of conditioned inactivation kinetics is an inactivation recovery experiment. A long prepulse which inactivates the sodium conductance is followed by repolarizing steps of various levels and durations to
allow varying degrees of recovery from the inactivated state. This is then followed by a standard test pulse to assess how much of the sodium conductance system has recovered to be reactivated. The results of such an experiment are shown in Fig. 9a. Over the range of potentials examined the
recovery time constants are larger in TNP. If Fig. 9a and b are viewed together as a unit, however, it appears that the slowing in recovery from inactivation results from a simple shift of the whole $\tau_c$ curve in the direction of hyperpolarization, in parallel with the shift of the $h_\infty$ relation.

It is also interesting to contrast the control values of $\tau_h$ and $\tau_c$ in the lobster axon membrane. In the voltage range below $-15$ mV a very large difference exists between the two values, $\tau_c$ being considerably larger than $\tau_h$ (Fig. 10). In all cases where $\tau_c$ and $\tau_h$ were compared on the same area of membrane this difference was observed. The values for the two time constants tend to

**Figure 10.** Comparison of control values for $\tau_c$ (filled circles) and $\tau_h$ (open circles). Mean $\pm$ SEM values for several axons plotted as a function of membrane potential. Both inactivation time constants were assayed in ASW as described in detail in Methods section.

merge for progressively larger depolarizations where $g_{Na}$ becomes fully activated.

**DISCUSSION**

The results of this study demonstrate that TNP specifically and reversibly alters the sodium conductance mechanism in lobster axon membranes in a complex manner. A simplifying summary of the action is that the voltage dependence of parameters governing the inactivation process assessed by conditioning voltage pulses are shifted in the hyperpolarizing direction, while the decay of the activated sodium conductance state is slowed for both maintained depolarizations and quick repolarizations. The alterations of sodium channel gating occur rapidly ($<1$ s) upon external application of TNP, while the potassium and leakage conductances remain unchanged. Details of the inactivation time-course at positive potentials and a more precise identification of possible small effects on $\tau_m$ are under investigation and will
be presented at a later time. Although our voltage clamp system was not compensated for possible series resistance, the near equality of peak current amplitudes in ASW and TNP effectively removes the possibility that the kinetic effects of TNP are merely a series resistance artifact.

Our finding of a large $\tau_c - \tau_h$ difference in normal axons at negative potentials is in agreement with the data of Goldman and Schauf, who reported similar differences in the two time constants in *Myxicola* giant axons and offered this as evidence for different processes governing normal and conditioned inactivation (Goldman and Schauf, 1973; Goldman, 1975). Quantitative aspects of $\tau_c - \tau_h$ differences should be viewed with some caution, however, due to the sensitivity of $\tau_h$ measurements to systematic errors, including uncompensated series resistance. H-H computer simulations of membrane currents with and without series resistance indicate that series resistance can reduce the apparent values of $\tau_h$, thus making $\tau_c$ appear larger in relative magnitude. The large $\tau_c - \tau_h$ differences in our data (Fig. 10) are considerably greater than what we estimate could result from a pure series resistance artifact, however.

The TNP data presented here are in basic agreement with the results of Cooke et al. (1968) who found that TNP reversibly increased the duration of propagated action potentials in lobster axons, but did not decrease spike amplitude or significantly alter resting potential. When TNP was applied to squid giant axons under voltage clamp conditions the potassium currents were not changed, while a slight reduction in $I_{Na}$ was observed over the voltage range investigated. Although data on ionic current kinetics was not reported, a clear increase in duration of the action potential from squid axons was observed suggesting a possible prolongation of $I_{Na}$. The reduction in peak sodium current amplitude on squid may possibly be attributed to a shift in the steady-state sodium inactivation curve, as observed in the present study, although no inactivation measurements were reported by Cooke et al. (1968).

Two Types of Inactivation

An interpretation of the present results requires a clear conceptual distinction between inactivation which occurs as the closure of open channels and inactivation which precedes and therefore prevents the opening of channels. For conditioning depolarizations which do not activate the majority of the sodium conductance (depolarizing potentials $<-20$ mV) inactivation kinetics ($\tau_c$) are clearly speeded up by TNP. For conditioning depolarizations which themselves activate the majority of the mechanism (depolarizing potentials $>-10$ mV) inactivation kinetics ($\tau_c$) are slowed by TNP, and the rate at which the activated component of the conductance mechanism decays during a maintained depolarization ($\tau_h$) is always slowed by TNP, regardless of what fraction has been activated.
The Hodgkin-Huxley model indicates a sodium channel of two independent components \((m\) and \(h\)) each of which can exist in an open or closed state, and with the requirement that both be open in order to allow sodium passage. Our results present a logical contradiction to this view of the gating mechanism, because they show that over the voltage range \(-50\) to \(-15\) mV the \(h\) process is speeded up by TNP when inactivation precedes activation but is simultaneously slowed when inactivation is measured as the decay of \(I_{Na}\) after activation. In addition our observation of a dichotomy between normal control values of \(\tau_h\) and \(\tau_s\) over much the same voltage range is at variance with the equality of the two time constants in the H-H formulation.

**An Alternative Conceptual Model**

As an alternative to the Hodgkin-Huxley model we propose a qualitative scheme of sodium channel gating behavior which is compatible with the TNP data and other experiments affecting gating properties. In this view, depicted in the diagram below, the sodium channel can exist in three basic states, resting, active, or inactivated, with transitory states between the basic states as represented by dots in the arrows.

Resting \(\rightleftharpoons\) Active
\[\rightleftharpoons\] Inactivated

At potentials near the normal resting potential most channels are in the resting state. A large depolarization converts the majority to the active (open) state. Maintaining the depolarization converts active channels to inactivated ones (coupled activation-inactivation) while repolarization converts the remaining active channels back to resting ones. On the other hand, smaller conditioning depolarizations which activate fewer channels can bring about direct inactivation from the resting state without coupling to activation. Such conditioned inactivation would reduce the population of channels available for activation by a stronger depolarization.\(^1\)

The modification of channel behavior by TNP is depicted in the alteration of the basic scheme shown below. The slowing of the closure of open channels is represented as the creation of an open substate which traps or holds activated channels temporarily in their open conformation. This slows the falling phase of current at all potentials to yield larger \(\tau_h\) values during maintained depolarizations and slows sodium tail currents when remaining active channels convert back to the resting state upon repolarization. An additional

\(^1\)A quantitative duplication of experimental behavior requires a specification of substates and rate constants, a refinement which does not change the basic nature of the scheme evolved from the data in this paper. Recently two quantitative kinetic models have appeared (Moore and Cox, 1975; Goldman, 1975) which also include dual inactivation routes as part of their structure.
modification of the basic scheme by TNP shifts the voltage dependence for

\[
\text{Resting} \xrightarrow{(\text{faster})} \text{Active} \xrightarrow{\text{Inactivated}} \]

the direct conversion of resting state to inactivated state in the direction of hyperpolarization. This shift, equivalent to that observed in the steady-state inactivation-voltage relation, increases the equilibrium fraction of channels in the inactivated state near the resting potential and yields lower values of \(\tau_e\) for small depolarizations. In contrast, large conditioning depolarizations (> -15 mV) which convert most channels to the active state (and prevent the direct inactivation process) yield a slower rate of conditioned inactivation (\(\tau_c\)) because the channels which open during the prepulse are also "trapped" in the active state by TNP. Consequently, the resultant time constant reflects mainly coupled activation-inactivation. Progressively smaller amplitude-conditioning depolarizations allow more and more channels to inactivate by the direct route (which is faster in TNP) and yield progressively lower values of \(\tau_c\) in TNP relative to controls.

The observation of significant increases in the amplitude of peak \(I_{Na}\) in TNP is similar to that seen upon application of *Condylactis* toxin to the nerve membrane (Narahashi et al., 1969). Although one would predict larger peak currents from the Hodgkin-Huxley model if TNP only increased \(h\), the observed increases of peak current were in all cases smaller than those calculated from the model using experimentally determined time constants. In the H-H system this would be interpreted as a decrease in \(g_{Na}\), the maximum sodium conductance. Such a conclusion, however, assumes that the \(h\) parameter decays from the onset of the voltage step. If inactivation were coupled to the activated state a delay in the inactivation process would occur such that in the absence of a decrease in functional channel population or of per channel conductance, the increase in peak \(I_{Na}\) caused by TNP would fall short of the upper limit predicted by the H-H model.

There exists a voluminous literature describing pharmacological alterations of gating processes in nerve membranes (see examples in Introduction). Although such modifications will indeed have to be accounted for in any quantitative refinement of the proposed qualitative scheme, a discussion of their relation to the proposed scheme without the benefit of a detailed kinetic model would be premature. We know of no pharmacological alterations, however, which are incompatible with the concept of dual inactivation mechanisms, one coupled to activation and the other uncoupled.

The experiments of Armstrong and Bezanilla describing possible gating currents are relevant to the present view in that gating currents are prevented if an attempt to elicit them is made while the sodium conductance is
inactivated (Armstrong and Bezanilla, 1974; Bezanilla and Armstrong, 1975). According to the H-H model this prevention should not occur, since the m process is independent of the state of h. The prevention is consistent with the proposed scheme, however, since significant activation (and presumably the gating currents associated with it) cannot occur with a state of large-scale inactivation. Progressive removal of inactivation (conversion of closed inactivated to closed resting channels) would allow progressively larger gating currents to be observed upon depolarization, a result confirmed experimentally (Armstrong and Bezanilla, 1974).

Although further experiments are required before the mode of action of TNP on the sodium channel can be defined at the molecular level, some statements can be made concerning possible mechanisms on the basis of present data. At the pH used in our experiments the phenolate anion species predominated ($pK_a = 0.38$) and in all likelihood is the active form of TNP. Considering the rather large shifts in the voltage dependencies of $\tau_v$ and $h_v$, an alteration of membrane surface charges resulting in an effective membrane potential more positive than the measured value might be involved in the TNP effect. In view of the rather selective effect upon sodium inactivation, however, a general change of diffuse double-layer potential would seem to be of only minor importance. The speed with which TNP exerts its effect on $\tau_v$ after a quasi-step exposure of the membrane suggests that no significant diffusion barriers exist between TNP molecules and their site(s) of action and that perhaps it acts at the external membrane surface. In view of recent evidence in support of the hypothesis that the sodium channel is protein in nature (see Armstrong et al., 1973, and Oxford and Poole, 1975), the ability of TNP to interact with a specific protein component of sodium channels may be important in its action. TNP can form dissociable charge-transfer complexes with substituted aromatic ring structures (Briegleb, 1961) and might interact with aromatic side chains of amino acids in membrane proteins. A classical protein precipitation reagent (Hamilton and Van Slyke, 1943), TNP could also form simple ionic bonds with basic amino acids. In any event we suggest that the slowing of channel closure by TNP results from interference with a normal voltage-sensitive change in protein configuration rather than a change in the effective membrane potential. Experiments designed to investigate these points are continuing at present.

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