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W. Ulbright and H.-H. Wagner

Phil. Trans. R. Soc. Lond. B 1975 **270**, 353-363
doi: 10.1098/rstb.1975.0015

Phil. Trans. R. Soc. Lond. B. **270**, 353–363 (1975) [353]
Printed in Great Britain

The reaction between tetrodotoxin and membrane sites at the node of Ranvier: its kinetics and dependence on pH

By W. Ulbricht and H.-H. Wagner
The Department of Physiology, University of Kiel, D-23 Kiel, Germany

Voltage clamp experiments were done on single nodes of Ranvier to study the inhibition of the sodium permeability by tetrodotoxin (TTX). Equilibrium results could be excellently fitted on the assumption that a sodium channel is blocked when one toxin molecule binds to it, the equilibrium dissociation constant, K_T of this reaction being 3.6 nM at 20 °C. Onset and offset of block could be quantitatively interpreted to be determined by the rates of the TTX-channel reaction whose average constants, at room temperature, were 3×10^6 M^{-1} s^{-1} for the association (k_1) and 1.4×10^{-2} s^{-1} for the dissociation (k_2). The dependence of the constants on temperature could be described by Arrhenius plots yielding activation energies of 29.3, 85.5 and 41.0 (57.3) kJ/mol for K_T, k_2 and k_1 (k_1 derived from onset alone), respectively. At low pH the relative TTX effect was clearly less than at neutral pH. These results could be explained by a model involving the competition of TTX and protons for the same receptor to which protons bind as a function of membrane potential.

Introduction

In myelinated nerve fibres of the frog as in most excitable cells in which the upstroke of the action potential is the result of sodium ions flowing into the cell, tetrodotoxin inhibits this sodium current very specifically and at very low concentrations (see reviews of Kao (1966) and of Evans (1972)). Interestingly the inhibition concerns only the amplitude of the sodium current while its kinetics and the general dependence on membrane potential remain unaffected (Hille 1968). If we adopt the commonly accepted view that this current flows through a finite number of uniform channels (see, for example, Hille 1970), a sufficient explanation of the inhibition would be that the toxin simply reduces the number of operating channels. The drug–receptor reaction underlying this block is of particular interest since one partner, the receptor, appears to constitute part of the sodium channel or is at least closely linked to it. The present paper describes two series of experiments dealing with this reaction. The first series was done in conjunction with J. R. Schwarz to analyse the rate of the toxin action (Schwarz, Ulbricht & Wagner 1973). The second series was done to study the influence of pH on the effect of tetrodotoxin and only short accounts of the results have so far been given (Wagner & Ulbricht 1973, 1974).

Equilibrium effects

Several years ago Hille (1968) described the equilibrium effects of tetrodotoxin on frog nerve fibres and his interpretation of the results can be expressed by the following scheme:

$$\mathrm{TTX} + \mathrm{R} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{TTX.R},$$

where one tetrodotoxin molecule, TTX, binds reversibly to one receptor, R, per channel to form a drug–receptor complex, TTX.R; k_1 and k_2 being the rate constants of the complex association and dissociation, respectively. The general implication is that the formation of TTX.R renders a channel completely impermeable so that, for example, a reduction of the sodium current to 80 % of its original value means that 20 % of the channels are blocked or, in other words, that the fractional receptor occupancy, y, is 0.2. Its equilibrium value, y_∞, depends on the analytical toxin concentration $c_T = [TTX]\, k_1/k_2$ as given by

$$y_\infty = c_T/(c_T + 1). \tag{1}$$

By definition, $c_T = 1.0$ for $[TTX] = k_2/k_1 = K_T$, i.e. the equilibrium dissociation constant of the reaction. At room temperature (20–23 °C) the mean K_T was 3.6 and 3.4 nM in the early experiments on *Xenopus laevis* and *Rana esculenta* nerve fibres, respectively (Schwarz *et al.* 1973). In the new series on *Rana* fibres, done between 16 and 18 °C with a different toxin sample, the mean K_T was 2.8 nM.

Rate of toxin action

While the equilibrium inhibition yields the ratio of the rate constants, their absolute values have to be determined in kinetic experiments. Their theoretical basis is the rate of receptor occupation derived from the simple reaction scheme given above. Hence if the toxin concentration in the immediate vicinity of the receptor is raised stepwise from zero to c_T, the occupancy follows from zero to y_∞ with an exponential time course governed by the onset time constant, τ_{on},

$$y = y_\infty(1 - e^{-t/\tau_{on}}), \tag{2}$$

with $1/\tau_{on} = [TTX]\, k_1 + k_2$. On washout the dissociation of the drug–receptor complexes results in the exponential decline of occupancy from its starting value, y_0, to zero:

$$y = y_0 e^{-t/\tau_{off}}, \tag{3}$$

where $1/\tau_{off} = k_2$. It should be noted that the offset proceeds independent of concentration in contrast to the onset that accelerates with increasing drug concentration. Another point worth mentioning is that k_1 can be calculated either with the help of both τ_{on} and τ_{off}, as is indispensable if only one toxin concentration is tested or from onset alone if two values of τ_{on} are determined at two different concentrations.

In our kinetic experiments we started from the assumption that the toxin–receptor reaction was limiting the rate of inhibition of the sodium current when the toxin was suddenly applied to the node of Ranvier. Because of the toxin's sole effect of reducing the current amplitude the development of this effect could be pursued by recording, in a voltage clamp experiment, the sodium current when cathodal pulses of constant amplitude were periodically applied to the membrane. An experiment of this kind is illustrated by figure 1 where the sodium current relative to its value in normal Ringer solution is plotted as a function of time. Two tetrodotoxin concentrations have been tested: 3.1 nM (open symbols) and 15.5 nM (filled symbols) and it is clear that the onset (circles) proceeded faster in the higher concentration. The curves through the points observed during the onset are exponential functions with τ_{on} of 45.0 and 17.4 s, respectively. The re-increase of the sodium current during washout, too, had an exponential time course but the time constants of the two curves were practically identical (τ_{off} = 76.5

and 78.0 s) as predicted by equation (3). In this experiment as in various others the relation of onset to offset time constants was fully compatible with the predictions of equations (2) and (3). This, however, is insufficient proof of the validity of our working hypothesis that the observed rates of action reflect the true reaction rates since in special cases of diffusion-limited binding to receptors quite similar relations can be found (Rang 1966; Thron & Waud 1968; Colquhoun & Ritchie 1972). This raised the general question to what extent our observed rates may have been determined by diffusion and, in particular, whether or not the toxin had ready access to the membrane.

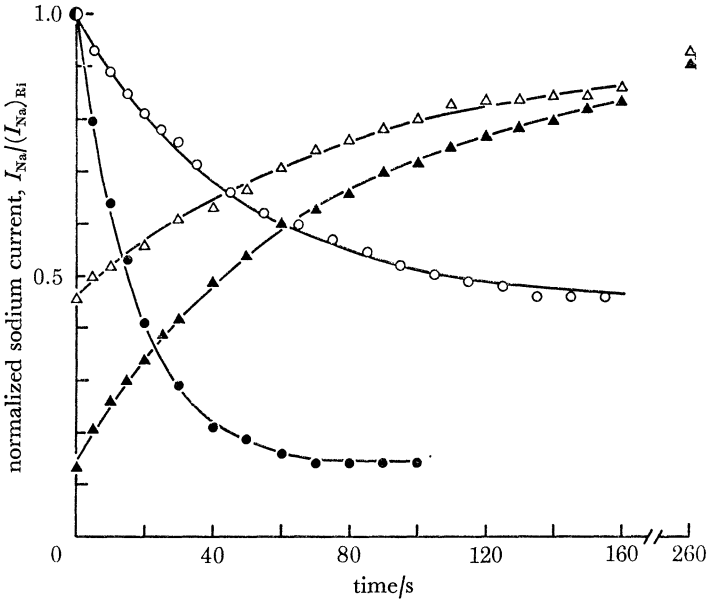

FIGURE 1. Development (circles) of and recovery (triangles) from TTX-induced inhibition of I_{Na} at room temperature. Ordinate: I_{Na} relative to its value before the application of 3.1 nM TTX (hollow symbols) or 15.5 nM TTX (filled symbols). Abscissa: time after the change of solutions. The curves were calculated with equations (2) and (3) with τ_{on} = 45.0 s (○) or 17.4 s (●) and τ_{off} = 76.5 s (△) or 78.0 s (▲). From Schwarz *et al.* (1973); by permission of the *Journal of Physiology*.

ACCESS TO THE MEMBRANE

To clarify this point experiments were done with the double air gap method that allows a very fast change of solutions perfusing the node of Ranvier. As a disadvantage this method does not permit an adequate voltage clamp during the flow of the early sodium current. Therefore the maximum rate of rise, $\dot{V}_A$, of the action potential was used as a measure of this current. In figure 2, $\dot{V}_A$ of a train of spikes (elicited every 40 ms) is plotted for a sudden change in perfusate from half the normal sodium concentration (59 mM) to its normal value in Ringer solution (118 mM). The ensuing increase in $\dot{V}_A$, as given by the triangles, was about 80 % complete after only 1.0 s and an even faster exchange can be achieved with this method (Vierhaus & Ulbricht 1971*a*). When this procedure was repeated with 118 mM Na + 155 nM TTX, a rather high toxin concentration, $\dot{V}_A$ quickly rose but soon decreased again (circles), obviously as the result of the toxin action. This action can be conveniently expressed by the difference between the two curves as shown by the interrupted curve below that set in about 0.1 s after the start of the Na effect. From the known details of the sodium exchange, the diffusion of the

accompanying tetrodotoxin can be estimated and it can be shown that the short latency in the blocking action is compatible only with an unrestricted access of the toxin to the membrane. This seems to exclude impeded bulk diffusion to be responsible for the observed slow rates of block.

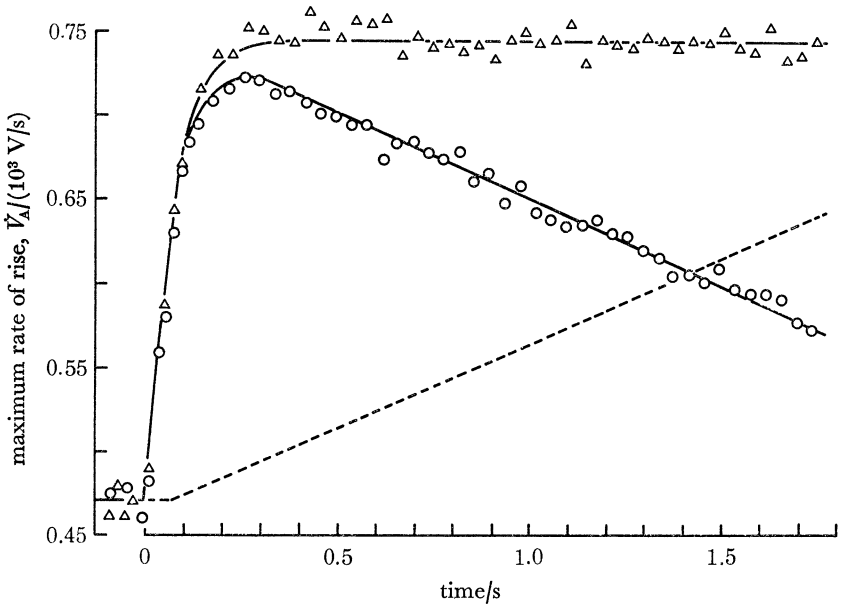

FIGURE 2. Latency of the effect of 155 nM TTX on the maximum rate of rise, $\dot{V}_A$, of action potentials elicited every 40 ms at 21 °C. Abscissa: time, in s, after the start of the sodium effect. Triangles refer to a change from 59 to 118 mM Na without toxin, circles refer to a change from 59 mM Na to 118 mM Na + 155 nM TTX. The continuous curves were drawn by eye and the difference between the two curves, the TTX effect, was given as an interrupted line. From Schwarz *et al.* (1973); by permission of the *Journal of Physiology*.

TEMPERATURE EXPERIMENTS

Additional information on the rate-limiting process was expected from temperature experiments that we did with 3.1 and 15.5 nM tetrodotoxin between 8 and 23 °C. From τ_{on} and τ_{off} determined in these experiments we calculated the rate constants k_1 and k_2 as outlined in connexion with equations (2) and (3). In figure 3 our results are shown in two semilogarithmic plots of the rate constants as a function of $1/T$. The upper of these Arrhenius plots contains k_1 as determined from the relation between τ_{on} and τ_{off}; the lower plot gives k_2. The values of either plot agree well with the mean rate constants of the preceding series at room temperature: $k_1 = 3.0 \times 10^6$ M^{-1} s^{-1} and $k_2 = 1.4 \times 10^{-2}$ s^{-1}. In this series the mean k_1 as calculated from onset alone had a comparable value: 3.3×10^6 M^{-1} s^{-1}.

From the slope of the regression lines in figure 3 the Arrhenius energy of activation was calculated to be 41.0 kJ/mol for k_1 (or 57.3 kJ/mol if derived exclusively from onset data) and 85.8 kJ/mol for k_2. In terms of Q_{10} as calculated for a step from 12 to 22 °C this would correspond to 1.8 (or 2.3) for k_1 and 3.4 for k_2. The different Q_{10} values for the two rate constants suggest that the *equilibrium* dissociation constant, K_T, should decrease on cooling. This was confirmed in equilibrium experiments that revealed a Q_{10} of 1.5 for K_T between 12 and 22 °C.

Although the temperature coefficients do not give unequivocal information about the rate-limiting process, lower Q_{10} values would be expected if the rate of action were determined by

diffusion to the nodal membrane. In squid axons, on the other hand, access to the membrane is impeded by a layer of Schwann cells and indeed the rate of toxin action was found not only to be slower but also less dependent on temperature (Cuervo & Adelman 1970).

Our present results on nodes of Ranvier appear to be satisfactorily explained by assuming that the observed rates of action are the true rates of the toxin-receptor reaction. Admittedly this reaction is unusually slow if compared, for example, to the blocking of potassium channels by tetraethylammonium ions in the same preparation (Vierhaus & Ulbricht 1971*b*). Before we start speculating, however, why this is so, more aspects of the toxin-receptor reaction should be studied. One aspect, the influence of pH, is dealt with in the following sections.

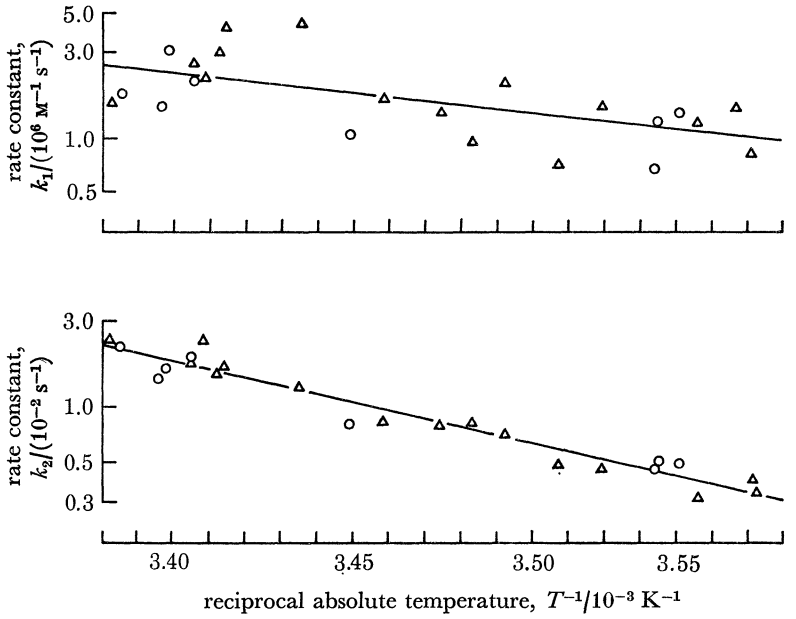

FIGURE 3. Dependence of the rate constants k_1 and k_2 on temperature. Arrhenius plot of data from ten experiments with either 3.1 nM TTX (○) or 15.5 nM TTX (△). Logarithmic ordinates in both panels; abscissae: reciprocal absolute temperature. The lines of regression were calculated as lg k_1 = 13.6737–2151 $(1/T)$, k_1 being expressed in $\mathrm{M^{-1}\ s^{-1}}$, and lg k_2 = 13.4892–4482 $(1/T)$, k_2 being expressed in $\mathrm{s^{-1}}$. From Schwarz *et al.* (1973); by permission of the *Journal of Physiology*.

COMBINED EFFECT OF H^+ IONS AND TETRODOTOXIN ON THE MAXIMUM RATE OF RISE

Figure 4 summarizes some of the interesting results observed with 15.5 nM tetrodotoxin whose effect on the maximum rate of rise, $\dot{V}_A$, of the action potential relative to its value in toxin-free neutral Ringer solution is plotted as a function of pH. The hollow circles are the mean values after equilibration in the respective solutions. Weakly acid toxin solutions were less effective than the neutral control as can be concluded from the re-increase in $\dot{V}_A$. This is intriguing since lowering the pH *per se* depresses $\dot{V}_A$ as indicated by the crosses referring to observations in toxin-free solutions. One immediately suspects a drastic decrease in the number of sodium channels blocked by tetrodotoxin at low pH. This was verified experimentally by $\dot{V}_A$ as measured 1 s after an abrupt change back to neutral Ringer solution. In separate experiments it was shown that after this time the block by protons was completely relieved while that by

the toxin was practically unchanged because of the large τ_{off}. The filled circles in figure 4 give the mean results of this procedure and reveal that the toxin's share in the combined effect is greatly reduced at low pH. Incidentally this conclusion agrees nicely with reports on reduced binding of labelled tetrodotoxin (and saxitoxin) at low pH (Benzer & Raftery 1972; Colquhoun, Henderson & Ritchie 1972; Henderson, Ritchie & Strichartz 1973). After inspection of figure 4 only one of the various possibilities of proton-tetrodotoxin interaction can be excluded right away, namely a model involving two separate and completely independent receptors for H^+ ions and toxin molecules for each channel where the occupation of one site suffices to block. Testing other models requires a quantitative analysis of the combined blocking effect for which $\dot{V}_A$ is not suited since it is doubly affected by H^+ ions: in the toxin-like manner that we are concerned with and by shifting the sodium permeability–voltage curve to more positive membrane potentials. The two effects, however, can be distinguished in voltage–clamp experiments.

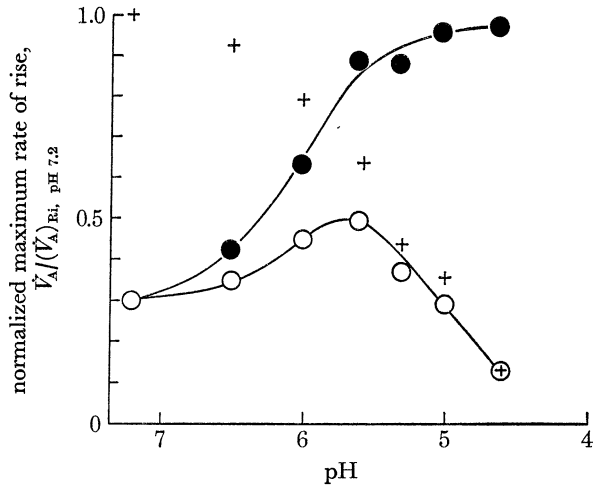

FIGURE 4. The effect of pH on the maximum rate of rise, $\dot{V}_A$, in 15.5 nM TTX and in TTX-free solutions at room temperature. Ordinate: $\dot{V}_A$ relative to its value in normal Ringer solution at pH 7.2; abscissa: pH. The points denote mean values after equilibration in 15.5 nM TTX (○), 1 s after changing back to neutral toxin-free solution (●) and after equilibration in TTX-free media of the respective pH (+). The curves were drawn by eye.

COMBINED EFFECT OF PROTONS AND TETRODOTOXIN ON THE SODIUM PERMEABILITY

A typical voltage-clamp experiment of this series is illustrated by figure 5 in which the peak sodium permeability, P_{Na}, is plotted as a function of membrane potential during the test pulses. The values of P_{Na} were calculated with the constant-field equation as suggested by Dodge & Frankenhaeuser (1959). The points observed in three runs in neutral Ringer solution (hollow circles, squares and diamonds) testify to the good reversibility so that they could be connected by a common curve that saturated for $E > 10$ mV. Addition of 9.3 nM tetrodotoxin to this neutral solution led to a downward shift of $P_{Na}(E)$. In this semi-logarithmic plot this means a reduction by a constant factor that was 0.25 (filled circles) corresponding to $y_\infty = 0.75$. This indicates that K_T was 3.1 nM for this preparation. When the node was tested in toxin-free solution at pH 5.6 the values of P_{Na} (hollow triangles) were found to be shifted to the right and to lower values and no saturation was seen. This characteristic P_{Na}–E curve in

toxin-free acid solution has been studied in detail by Woodhull (1973) who assumed a voltage-dependent block of sodium channels as the result of a very fast binding of H^+ ions. The idea is that with increasing depolarization fewer channels remain blocked so that the curve through the hollow triangles approaches the curve observed in neutral Ringer solution. The P_{Na} values in acid solution can be corrected for voltage-dependent blocking so that for $E > 10$ mV they become identical with the values in neutral Ringer solution. For more negative potentials the corrected permeabilities are indicated by the interrupted curve which now reveals the true pH-induced shift along the voltage axis, 13 mV in this case.

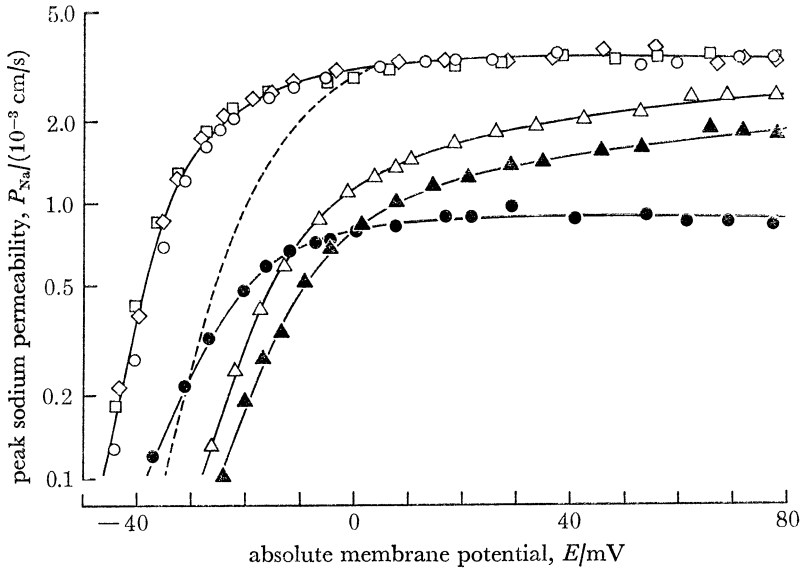

FIGURE 5. Peak sodium permeability as a function of membrane potential in 9.3 nM TTX and in toxin-free solution at pH 7.2 or 5.6. Ordinate: peak sodium permeability, P_{Na}, in 10^{-3} cm/s on a logarithmic scale. Abscissa: absolute membrane potential, E, during the test pulses, in mV, depolarization to the right. Points ○, ◇ and □ were observed in Ringer solution of pH 7.2 during the 1st, 4th and 6th run; ● in 9.3 nM TTX at pH 7.2 (2nd run), ▲ in 9.3 nM TTX at pH 5.6 (3rd run) and △ in toxin-free solution of pH 5.6 (5th run); 16 °C throughout. Interrupted curve after correction of △ for voltage-dependent binding of H^+ ions.

When 9.3 nM tetrodotoxin was added to the acid solution (filled triangles) the P_{Na}–E relation was reduced by a constant factor and hence shifted downward in this plot. This curve, after correction for constant factor and for the proton-induced block, revealed approximately the same amount of shift along the E axis. In our various experiments with different toxin and proton concentrations we were unable to detect a significant influence of the toxin on the proton-induced shift.

A MODEL OF PROTON–TOXIN COMPETITION FOR THE SAME SITE

In searching for a model to fit our observations in acid toxin solutions we have also tested the tempting idea that protons and toxin molecules compete for the same receptor whose occupation results in the occlusion of the channel. The theoretical basis is as follows. Let

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y'_∞ be the total occupancy at equilibrium and in the presence of both toxin and protons, each contributing its share, y'_T and y'_H. We then have

$$y'_\infty = y'_T + y'_H = \frac{c_T}{c_T + c_H + 1} + \frac{c_H}{c_T + c_H + 1}, \tag{4}$$

where c_T is the analytical toxin concentration as defined on page 354 and $c_H = [H^+]/K_H(E)$ is the analytical hydrogen concentration. Since blocking by protons is voltage-dependent the equilibrium dissociation constant K_H must be a function of membrane potential E. This fact is of particular importance since we have to realize that equilibrium with toxin molecules and protons takes place at the holding potential, E_{HP} (usually the resting potential of -70 mV) and that $c_H(E_{HP})$ influences both y'_H and y'_T. Hence we should describe this situation by

$$y'_\infty(E_{HP}) = y'_T(E_{HP}) + y'_H(E_{HP}) \tag{4a}$$

although it cannot directly be tested since the gates of the sodium channels are closed at E_{HP}. When we apply a cathodal test pulse to open the gates we also change the situation with respect to the pharmacological block since the proton-site reaction is so fast that a new equilibrium is reached soon after the make of the pulse (Woodhull 1973) and $y'_H(E_{HP})$ reduces to $y'_H(E_t)$. The toxin-site reaction, on the other hand, is much too slow so that channels blocked by the toxin at E_{HP} remain so during the test pulse and so does $y'_T(E_{HP})$. The total occupancy at $E = E_t$ then is

$$y'(E_t) = y'_T(E_{HP}) + y'_H(E_t). \tag{4b}$$

To derive $y'_H(E_t)$ we realize that each of the two blocking agents equilibrates with the channels left free by the other agent. Therefore we can write

$$y'_H(E_t) = \frac{c_H(E_t)}{c_H(E_t) + 1}\,[1 - y'_T(E_{HP})]. \tag{5}$$

If we now denote $p'_T(E_{HP}) = 1 - y'_T(E_{HP})$ and $y_H(E_t) = c_H(E_t)/[c_H(E_t) + 1]$, we obtain

$$y'_H(E_t) = y_H(E_t)\, p'_T(E_{HP}). \tag{5a}$$

We further introduce equation (5a) into (4b), denote $p'(E_t) = 1 - y'(E_t)$ and solve for this complement

$$p'(E_t) = p'_T(E_{HP}) - y_H(E_t)\, p'_T(E_{HP}). \tag{6}$$

Finally, since $1 - y_H(E_t) = p_H(E_t)$, we obtain

$$p'(E_t) = p'_T(E_{HP})\, p_H(E_t). \tag{7}$$

The p's are directly applicable to our results since they give the fractions of channels left unoccupied under the respective experimental circumstances and hence are proportional to P_{Na}. Equation (7) then shows that for a given value of E_t, P_{Na} in acid toxin solution equals P_{Na} in toxin-free solution of the same pH *times* a constant factor, i.e. $p'_T(E_{HP})$ as it was indeed observed (see figure 5). Moreover, equation (7) predicts that this factor should depend on the holding potential.

Testing the model of competition

Figure 6 illustrates an experiment that was done at pH 5.2 throughout to test the prediction from the model. In each panel the peak sodium permeability, P_{Na}, is plotted on a logarithmic scale as a function of the potential during the test pulses; hollow triangles give P_{Na} in the

toxin-free solution while the filled triangles refer to P_{Na} in acid toxin (15.5 nM) solution. The only difference is that the upper pair of curves was obtained after the preparation had been equilibrated at $E_{HP} = -50$ mV so that hyperpolarizing prepulses had to precede the test pulses to remove sodium inactivation; 50 ms pulses to $E = -80$ mV were sufficient at this low pH. The lower pair of curves was observed with $E_{HP} = -90$ mV so that no prepulses were necessary. The influence of the holding potential was obvious as can be seen by the vertical distance between the curves of each pair. The corresponding p'_T was 0.74 for $E_{HP} = -50$ mV and 0.82 for $E_{HP} = -90$ mV. The respective mean values ($\pm$s.e.m.) of four fibres were 0.60 ± 0.05 and 0.79 ± 0.04, i.e. even more distinctly different. The result was as predicted since during a steady hyperpolarization to $E = -90$ mV more channels were occupied by protons and consequently fewer by the toxin so that the additional inhibiting effect of TTX, as revealed on testing, was diminished (see lower part of figure 6). In separate experiments at neutral pH where $c_H \approx 0$ we were unable to detect voltage-dependent binding. This indicates that, as assumed in the model, the dependence on E_{HP} resides only in $c_H(E)$.

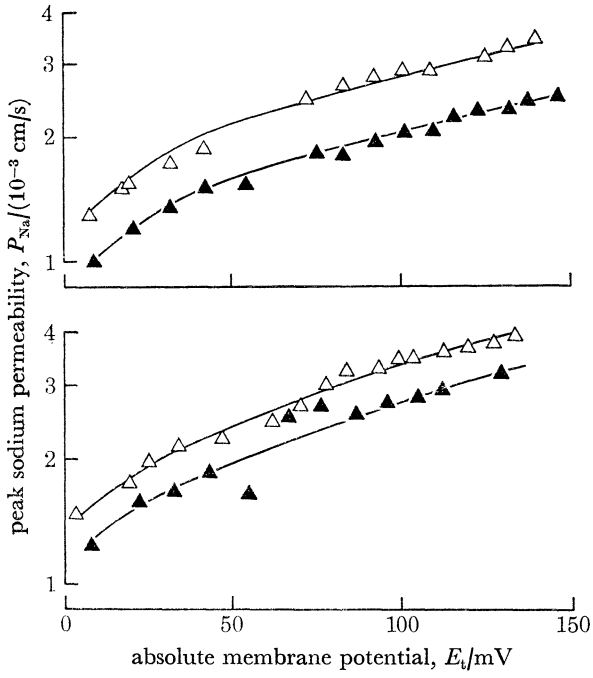

FIGURE 6. Influence of holding potential on additional inhibiting effect of 15.5 nM TTX at pH 5.3. Ordinates: peak sodium permeability, P_{Na}, in 10^{-3} cm/s, logarithmic scale. Abscissae: absolute membrane potential, E_t, during the test pulses, in mV. Observations after equilibration at a holding potential, E_{HP}, of -50 mV (upper panel) and $E_{HP} = -90$ mV (lower panel). In both plots △ refers to P_{Na} in toxin-free acid solution. Addition of 15.5 nM TTX (▲; also at pH 5.3) reduced P_{Na} by a constant factor, p'_T, resulting in a downward shift of the curves (drawn by eye); p'_T was 0.74 and 0.82 in the upper and lower panel, respectively. Temperature: 16 °C.

While the model thus gave a good qualitative description, the quantitative fit was less satisfactory since the predicted values of p'_T were consistently too low as if we would have overestimated the weight of the toxin effect. This could indeed have been the case. It has repeatedly been proposed that the shift in the P_{Na}–E curve at low pH (see figure 5) reflects the reduction of a negative potential at the outer membrane surface. This in turn would reduce the concentration of cations (including the singly charged tetrodotoxin) in the double layer. Hence at

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low pH the effective toxin concentration immediately outside the membrane would be lower than at neutral pH and could thus be overestimated. A mechanism of this kind has been assumed by Mozhaeva & Naumov (1972) for the effect of 1 pH on the block of potassium channels by tetraethylammonium ions and we are grateful to Dr B. Hille for suggesting this possibility.

TABLE 1. TETRODOTOXIN EFFECT AT LOW pH – OBSERVED AND CALCULATED VALUES

		calculated			observed		
pH	[TXX] nM	c'_T†	$c_H(E_{HP})$‡	$p'_T(E_{HP})$§	$p'_T(E_{HP})$ mean ± s.e.	ΔE‖ mV	n
6.0	3.1	0.80	1.86¶	0.78	0.76 ± 0.02	8.7	3
5.6	9.3	1.81	3.54††	0.71	0.77 ± 0.02	15.3	3
5.3	15.5	2.79	6.19‡‡	0.72	0.72 ± 0.04	17.4	5

† Value of c_T corrected for effect of reduced surface potential: $c'_T = c_T \exp(\Delta EF/RT)$, where c_T = [TTX]/2.8 nM.
‡ Calculated for $E_{HP} = -70$ mV from $c_H(E) = [H^+]/K_H(E)$.
§ $p'_T(E_{HP}) = 1 - y'_T(E_{HP}) = [c_H(E_{HP}) + 1]/[c_H(E_{HP}) + c'_T + 1]$ with $E_{HP} = -70$ mV; see equation (4).
‖ Mean pH-induced shift of $P_{Na}(E)$.
¶ From observed mean $K_H(E) = 1.84$ μM exp (0.44 EF/RT).
†† From observed mean $K_H(E) = 1.88$ μM exp (0.35 EF/RT).
‡‡ From observed mean $K_H(E) = 2.05$ μM exp (0.33 EF/RT).

It should be mentioned, however, that this mechanism alone could not account for our findings nor can we at present prove its existence. Anyway, with this correction the calculated values of $p'_T(E_{HP})$ agreed nicely with the mean observed values as shown in table 1. We are aware that the good agreement could be fortuitous and as such neither proves nor disproves the competition model. Our model, therefore, should be considered tentative and it may be too simple as suggested by kinetic experiments at low pH that are currently being carried out in our laboratory.

We should like to thank Miss E. Dieter for unfailing help with the figures and throughout the experiments. Support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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