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Use-dependent block with tetrodotoxin and saxitoxin at frog Ranvier nodes

I. Intrinsic channel and toxin parameters

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Abstract. The use-dependent phasic blockage of sodium channels by tetrodotoxin (TTX) and saxitoxin (STX) was examined in frog nodes of Ranvier using trains of depolarizing pulses. The decline of the peak Na^+ current from its initial value (I_0) before the train to a stationary value (I_∞) after the train was more pronounced at more negative holding potentials. The relationship between I_∞/I_0 and holding potential was fitted by a sigmoid function which yielded values for the steepness of the voltage dependencies of around −15 mV for TTX and −8 mV for STX. Similar values were obtained at toxin concentrations of 4 and 8 n*M*. The higher voltage sensitivity of STX versus TTX is interpreted in terms of the higher charge and the faster binding kinetics of STX. These differences also explain the frequency dependence of the decline of Na^+ currents with STX (between 0.5 and 2 Hz) and the frequency independence with TTX. Variation of the pulse amplitude in a train of conditioning pulses revealed that the magnitude of the use-dependent actions of STX parallels the steady-state Na^+ inactivation curve h_∞. Inhibition of inactivation, by pre-treatment with chloramine-T, did not, however, abolish the use dependence. Instead, it introduced a change in the time constants of the decline of the Na^+ currents and the magnitude became independent of the holding potential.

Key words: Na^+ channel – Use dependence – Tetrodotoxin – Saxitoxin – Myelinated nerve

Introduction

It was recently shown (Salgado et al. 1986; Lönnendonker 1989; Patton and Goldin 1991) that use-dependent phasic effects for guanidinium toxin block of Na^+ channels in nerve can be induced by hyperpolarizing holding potentials. Interestingly, the divalent STX had a higher voltage sensitivity than the monovalent TTX. This and a following paper present more experiments and extend the analysis of these effects. The kinetic details of the process of use dependence were described by a model (Lönnendonker 1989) which included the kinetics of tetrodotoxin (TTX) or saxitoxin (STX) block of Na^+ channels (Ulbricht and Wagner 1975; Wagner and Ulbricht 1975). In addition, it was assumed that a depolarizing pulse triggers an increase in the toxin affinity which then slowly relaxes to the original value. However, the factors which modulate toxin affinity were unknown.

In this paper, I examine the different voltage sensitivities of use dependence of both toxins and the influence of the toxin concentration on this dependency. Moreover, it will be shown that the inactivation of Na^+ channels is an important parameter in determining the magnitude of use-dependent effects. Inhibition of inactivation by chloramine-T, however, did not simply abolish the use-dependent decline of Na^+ currents.

Some of these results have been published in abstract form (Lönnendonker 1991 a). The effects of various external cations on the use-dependent toxin block will be treated in the following paper (Lönnendonker 1991 b).

Materials and methods

Single motor fibres dissected from the tibial nerve of the frog *Rana esculenta* (Stämpfli and Hille 1976) were mounted in a nerve chamber. The node in the central compartment was continuously superfused by external solutions at 15 °C and voltage clamped (Nonner 1969). K^+ currents were blocked. At the beginning of the experiment the membrane potential was adjusted in Ringer's solution to obtain a steady-state Na^+ inactivation equivalent to $h_\infty = 0.7$, and deviations from this potential are denoted by *V*. The majority of the linear components of leakage and capacity currents were compensated by an analogue circuit. The series resistance (not compensated) was small because all external solutions contained toxin to block a

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significant amount of the sodium current. All voltage axes were not corrected for differences in junction potentials.

Solutions

The external solution contained 110 m*M* NaCl, 2 m*M* $CaCl_2$, 10 m*M* tetraethylammonium chloride (TEA-Cl), 4 m*M* morpholinopropane sulfonic acid (MOPS). To this solution different amounts of tetrodotoxin (TTX, Sigma) or saxitoxin (STX, a gift from Prof. J. M. Ritchie, Yale University) were added. The internal bath solution contained 113 m*M* CsCl, 7 m*M* NaCl, 4 m*M* MOPS. The pH of all solutions was adjusted to 7.2 with NaOH, thereby increasing the Na^+ concentration by 3 to 4 m*M*.

Measurements

The setting of the holding potential, the application of all voltage pulses and the current recording were done under computer control (LSI 11/73; Hof 1986). After the onset of the test pulses, the currents were sampled for 12 ms at two sampling intervals (10 μs up to 2 ms, 100 μs at later times). The computer program allowed test pulses to be applied repetitively (normally at 1 Hz) to $V = 60$ mV to elicit use-dependent effects. Other pulse protocols are described in the Results section. After a change of the holding potential or after a train of pulses an interval of at least 5 min was allowed in order to attain a steady-state.

The first peak Na^+ current, which is not influenced by a use-dependent decline, (the control) is denoted by I_0 and all other peak currents after the time t by I_t. The I_t values normally denote means of several subsequent peak current values (see figure legends). For the time of the mean value, the time of the last pulse in the group was chosen and denoted as t_p in the figures (Lönnendonker 1989).

Fitting was accomplished by the method of least squares (Zurmühl 1965). The data of the decline of peak currents from the experiments with repetitive pulsing were fitted with one exponential (I_∞ denotes the steady-state value, τ is the time constant and t the pulse time t_p):

$$I_t/I_0 = (1 - I_\infty/I_0) \cdot \exp(-t/\tau) + I_\infty/I_0 \,. \quad (1)$$

To describe data with only small changes of the I_t/I_0 amplitude it was necessary to use a fixed time constant τ in the fitting process. This τ was chosen to be in the range of the time constants found within the same paradigm. From these fits only the parameter I_∞ was used in the analysis.

The relationship between the ratios I_∞/I_0 and the holding potential V_H were analysed by two different methods. The linear part of the dependencies could be described by a regression line and the decline magnitude given in I_∞/I_0 per mV. This allowed confidence limits to be given for the slopes to enable a better comparison between different data sets. In addition, it was assumed that the curves are sigmoid, and the ratios were fitted by

$$C(V) = [1 - y]/[1 + \exp((v - v_{mid})/s)] + y \,, \quad (2)$$

where v is the holding potential V_H, y the end value reached at hyperpolarizing holding potentials, v_{mid} the inflection point and s the steepness factor of the curve.

The Hodgkin-Huxley parameters m_∞ and h_∞ of steady-state Na^+ activation and inactivation were obtained by conventional methods (e.g. see Neumcke and Stämpfli 1982). Linear regression analysis and calculation of the 95% confidence limits were performed by methods from Stange (1971).

Results

Differences between TTX and STX

The influence of the toxin charge on the use dependence and its relation to the holding potential is of interest. Salgado et al. (1986) mostly reported results using the divalent STX and Lönnendonker (1989) tested only one concentration of STX or the univalent TTX. To enable a direct comparison with data from newer experiments presented here and in a following paper (Lönnendonker 1991 b) the experiments were repeated with a test frequency of 1 Hz and two TTX and STX concentrations. The use-dependent effect could be characterized by a fit of the decline of the normalized currents (Eq. (1)) yielding a steady-state value I_∞/I_0 and a time constant τ. The absolute values of I_∞/I_0 at $V_H = -40$ mV and in solutions containing TTX (STX) were 0.84 ± 0.019 (0.66 ± 0.022) for 4 n*M* and 0.83 ± 0.020 (0.68 ± 0.029) for 8 n*M*. The steepnesses of the relationship between I_∞/I_0 and V_H found with (2) were -17.15 (4 n*M* TTX) or -12.33 mV (8 n*M* TTX) and -7.21 (4 n*M* STX) or -9.23 mV (8 n*M* STX). The regression analysis gave slopes of 0.0032 ± 0.0022 I_∞/I_0 per mV (slope ± 95% confidence limit) for 4 n*M* TTX and 0.0036 ± 0.0028 I_∞/I_0 per mV for 8 n*M* TTX. The experiments with STX gave slopes of 0.012 ± 0.0015 I_∞/I_0 per mV for 4 n*M* STX and 0.015 ± 0.0035 I_∞/I_0 per mV for 8 n*M* STX. Thus the differences between the toxin concentrations of 4 and 8 n*M* were negligible. The fitted time constants τ show a larger scatter. In the following it is assumed that τ was nearly independent of the holding potential. The mean time constants τ were 39.52 ± 1.5 and 27.03 ± 2.1 s for TTX and 13.35 ± 1.2 and 6.98 ± 0.68 s for STX, with 4 and 8 n*M* toxin respectively. Thus a higher toxin concentration induced a lower time constant and TTX produced use-dependent effects which were three times slower.

Frequency of test pulses

In crayfish axons the decline of Na^+ currents in STX solutions is strongly dependent on the gap time between pulses in a train (Salgado et al. 1986). This was also found for the use-dependent declines of Na^+ currents at the node of Ranvier of the frog. Figure 1 shows the results with different frequencies of test pulses in solutions with 8 n*M* STX. The pulse gaps were varied between 0.5 and 2 s. Additionally, no influence on the time constant, τ, was found (Fig. 1 B). In contrast, the TTX results (not shown)

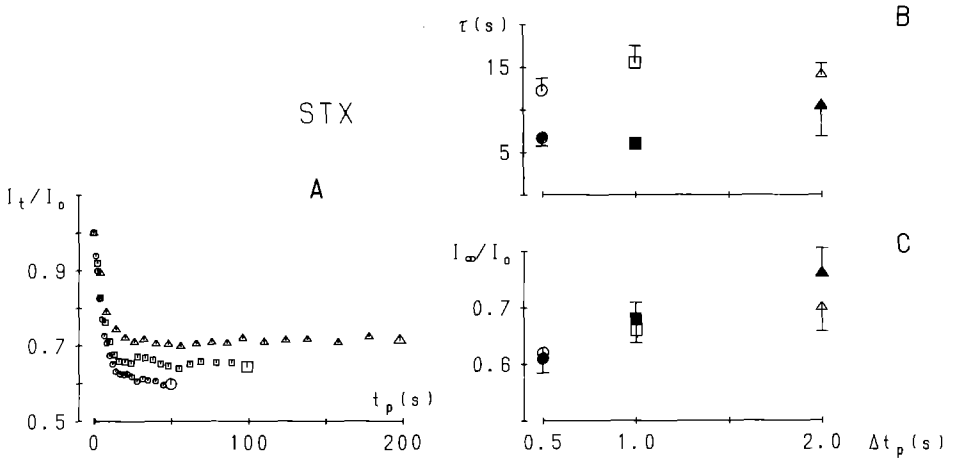

Fig. 1 A–C. Frequency dependence of Na^+ currents in Ringer with 4 n*M* (open symbols) and 8 n*M* (filled symbols) STX. **A** Normalized peak currents I_t/I_0 vs. pulse time t_p with 4 n*M* STX for three different frequencies (o 2 Hz, □ 1 Hz and △ 0.5 Hz). The last value for each experiment is plotted with a larger symbol. From such single experiments parameters were determined by exponential fits. **B** Shows the time constant of decline, τ and (**C**) the normalized stationary current, I_∞/I_0 (means ±SEM) vs. pulse interval Δt_p. Holding potential $V_H = -40$ mV

were not influenced in the narrow range of high pulse frequencies used.

Figure 1 A clearly shows that the declines of peak currents in STX containing solutions at pulse frequencies of 2 Hz reached a lower steady-state level than at 1 Hz. Thus the magnitude of the decline in peak amplitude in experiments with solutions containing 8 n*M* STX or TTX at pulse frequencies of 1 Hz are difficult to compare.

It was assumed that the onset of use-dependent block during rapid stimulation would be limited by the rate of binding of toxin (Salgado et al. 1986). Figure 1 B shows that in 4 (open symbols) and 8 n*M* STX (filled symbols) the time constants τ were around 12 and 6 s respectively. The time constants of development of use dependence are therefore proportional to the toxin concentration. Obviously for these external solutions the assumption is true (see Discussion).

Conditioning the use dependence

The potential which was sufficient to obtain an increase in phasic toxin block was examined in solutions containing 8 n*M* STX and with trains of pulses with 1 s interpulse intervals. The first pulse of the train had a test amplitude of 60 mV and elicited a current not influenced by any use dependence. After this pulse, 40 pulses of amplitude, V_p, were applied. A train of 40 pulses to $V_p = 60$ mV would normally be sufficient to yield a stationary amplitude. In various experiments, V_p was between −10 and +70 mV. Figure 2 depicts the currents in response to test pulses before and after the trains of conditioning pulses with three different V_p amplitudes as indicated. Pulses to $V_p = -10$ mV from the holding potential $V_H = -30$ mV (Fig. 2 A) did not produce any use dependence: the current amplitudes initiated by test pulses to 60 mV after the conditioning train clearly showed the same decline as without conditioning. In contrast, a train of pulses to $V_p = 20$ mV (Fig. 2 C) elicited maximal use-dependent effects and no further decline was observed after the end of the train. Conditioning pulses to $V_p = 0$ mV (Fig. 2 B) produced submaximal use-dependent effects.

From such experiments, a decline ratio I_∞/I_1 was extracted where I_1 is the peak current amplitude produced

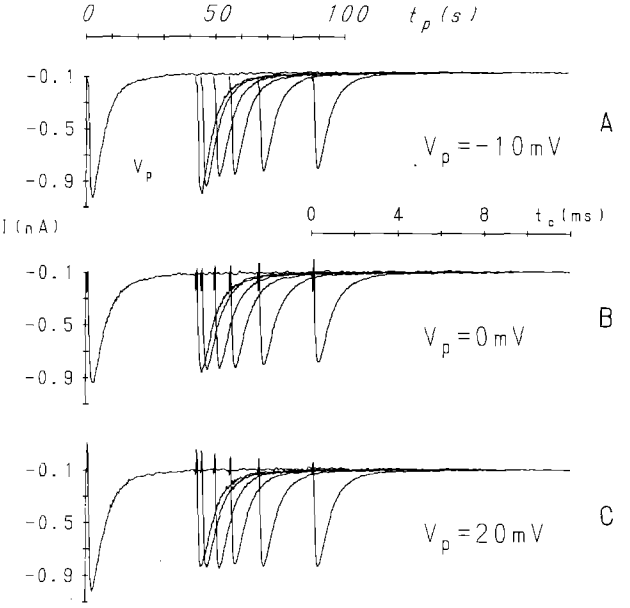

Fig. 2 A–C. Na^+ currents during trains of depolarizing test pulses ($V = 60$ mV) with an interval of 40 pulses to $V_p = -10$ mV (**A**), 0 mV (**B**) or 20 mV (**C**). The currents produced by V_p are not shown. The first pulse to $V = 60$ mV elicits currents without use-dependent effects and the currents remaining after the pulses to V_p show that there was a use-dependent action at $V_p = 20$ mV (**C**) or no use-dependent effect at $V_p = -10$ mV (**A**). The currents after the pulse time t_p are displaced against each other. The time axis labelled t_c gives the time for each individual recording. Ringer solution with 8 n*M* STX, holding potential $V_H = -30$ mV, pulse frequency 1 Hz and duration of all pulses 12 ms

by the first test pulse to 60 mV after conditioning pulse train. From these relative amplitudes the means were calculated for each conditioning pulse amplitude V_p and these means normalized between 0 and 1 and inverted, giving r_{use} (explained in legend to Fig. 3). In Fig. 3 A these r_{use} values are shown vs. the conditioning pulse amplitudes V_p. The figure also shows a fit of a saturation curve to the data. The means of the steady-state Na^+ inactivation, h_∞ and Na^+ activation, m_∞ values from another series of experiments are shown in Fig. 3 B. The fit curve from Fig. 3 A is repeated in Fig. 3 B and clearly describes the inactivation values of the Hodgkin-Huxley parame-

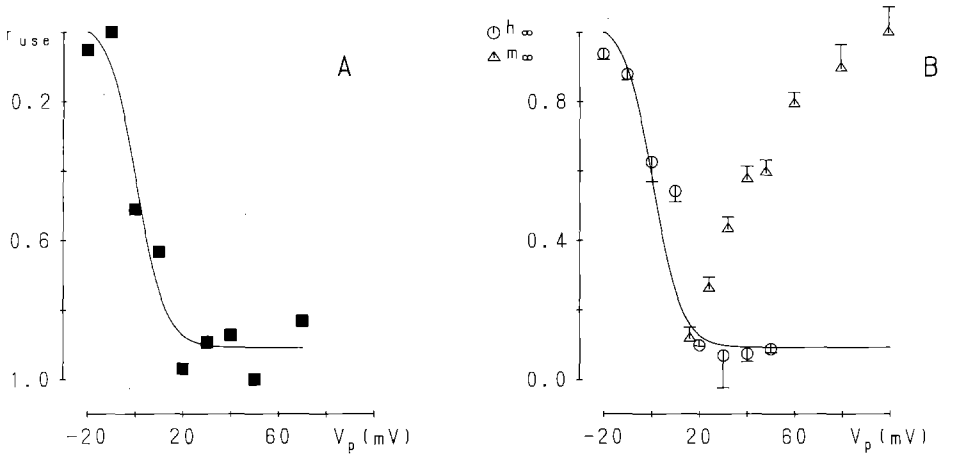

Fig. 3 A, B. Influence of inactivation and activation on use dependence. From results as shown in Fig. 2 the remaining normalized stationary currents, I_∞/I_1 after the pulse train to V_p and their means for each V_p, r_V, were calculated. r_V varies between 0.7 and 1.0 and the values were therefore normalized between 0 and 1 by $r_V^* = (r_V - 0.7)/(1 - 0.7)$ and then inverted to $r_{use} = 1 - r_V^*$. r_{use} is 0 if V_p produces no detectable use-dependent effects and 1 if it produces maximal use-dependent actions. r_{use} is shown in **A** vs. the pulse height V_p; note the inverted ordinate scaling. The curve is given by:

$$r_{use}(V_p) = 0.94 - [0.94/\{1 + \exp((V_p - 0.6)/6.0)\}].$$

This curve is repeated in **B** with the Hodgkin-Huxley values (mean ± SEM) of steady-state activation m_∞ (symbol △) and inactivation h_∞ (symbol ○) from 4 fibres. Holding potential $V_H = -30$ mV, pulse frequency 1 Hz for the experiments in **A**

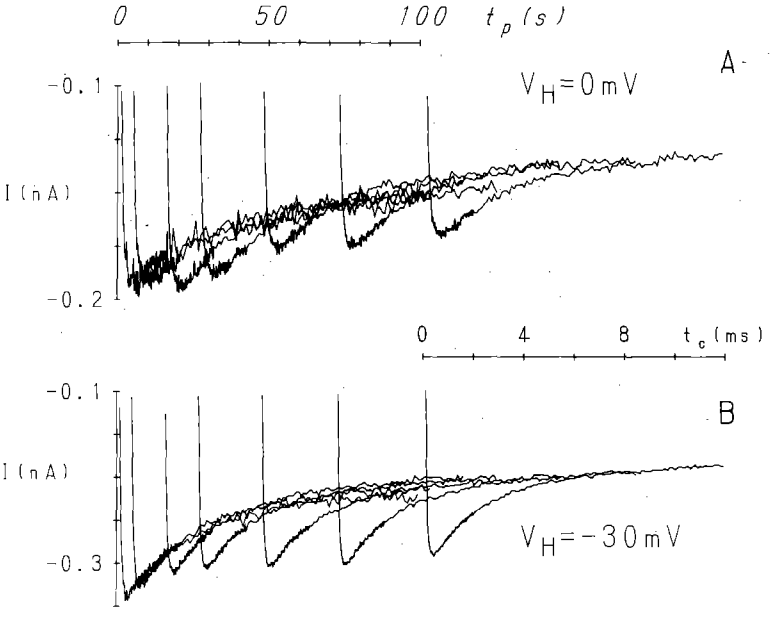

Fig. 4 A, B. Na^+ currents during a train of depolarizing pulses ($V = 60$ mV, pulse frequency 1 Hz) after removing a large part of inactivation by 6 min lasting pre-treatment with 0.5 m*M* chloramine-T. Currents are recorded in Ringer with 8 n*M* STX after washout of chloramine-T at two different holding potentials $V_H = 0$ mV (**A**) and $V_H = -30$ mV (**B**). The time axis t_c shows the kinetics of the currents and t_p the pulse time

ters. Thus pulse amplitudes which produce inactivation of Na^+ channels were sufficient to induce use-dependent actions. An activation of the channels was not necessary.

Inhibition of Na^+ inactivation

This striking dependence of the use dependence on Na^+ inactivation was examined in another series of experiments. If channel inactivation induced the use dependence, it was of interest to study the effect in channels in which inactivation was inhibited. This was achieved by a 6 min exposure to a Ringer's solution with 0.5 m*M* chloramine-T. Figure 4 shows Na^+ currents after the pre-treatment in Ringer containing 8 n*M* STX produced with a train of pulses to $V_p = 60$ mV. The holding potential was set to $V_H = 0$ mV (Fig. 4 A) or to $V_H = -30$ mV (Fig. 4 B). Despite the partial inhibition of Na^+ inactivation by chloramine-T, the decline of the currents was large and it could clearly be seen even at a holding potential of $V_H = 0$ mV.

As shown in Fig. 5, following chloramine-T pre-treatment I_∞/I_0 became independent of the holding potential, whereas τ became dependent on the holding potential. This is contrary to the relations seen with untreated fibres which are depicted by linear regression lines in Fig. 5. Moreover, the currents in Fig. 4 show that inactivation remained in only 1/3 of the Na^+ channels (mean: 28%),

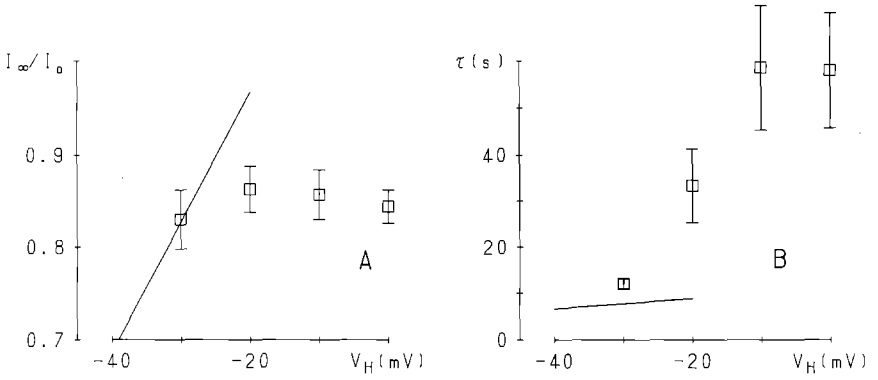

Fig. 5 A, B. Use-dependent block with STX after modification of inactivation. From results as shown in Fig. 4 the values for the decline of Na^+ currents during the train of pulses was extracted. The normalized stationary current, I_∞/I_0 and the time constant, τ of this decline (means $\pm$SEM) are shown vs. the respective holding potential V_H. Also depicted are the regression lines for the values without modification of inactivation in the same solutions (Ringer with 8 n*M* STX)

whereas the magnitude of the decline depicted in Fig. 5 A was only slightly reduced compared to the value at $V_H = -30$ mV in untreated fibres. This holds true even for an experiment with 75% remaining inactivation. Control experiments after chloramine-T treatment without toxin showed no significant decline during a train of depolarizing pulses at $V_H = -30$ mV.

Discussion

Qualitative description

A qualitative description of the decline of Na^+ currents induced by repetitive pulsing can be given in the framework of a previously proposed model (Lönnendonker 1989): Each pulse of the train increases the overall affinity of Na^+ channels for the guanidinium toxins. The affinity increase then relaxes slowly until the onset of the subsequent test pulse, thus allowing toxin molecules to bind to and block more channels than before. The increase in use-dependent effects at more hyperpolarized holding potentials can be partly explained by interactions between external Ca^{2+} ions and toxin molecules (Salgado et al. 1986; Lönnendonker 1991 b). Some of the implications of this model are analysed in detail below.

The time constants of decline

The time constants τ of the use-dependent decline of peak Na^+ currents can be compared with the time constant τ_{on} of the onset of toxin reaction as taken from Ulbricht and Wagner (1975) and Wagner and Ulbricht (1975). If a rapid change of the number of the free toxin binding sites with each triggering pulse is assumed this should produce comparable results for τ_{on} and τ. Indeed, there is reasonable agreement between the time constants for TTX: $\tau_{on} = 38.5$ s (26.3 s) and $\tau = 39.52$ s (27.03) for 4 (8) n*M*. However, for STX the values for use-dependent decline were smaller than the corresponding τ_{on} values: $\tau_{on} = 17.0$ s (10.2 s) and $\tau = 13.35$ s (6.98 s) for 4(8) n*M*. Such kinetic differences between both toxins were also found in two-pulse experiments (Lönnendonker 1989). The identical temporal behaviour in TTX solutions can be interpreted by the assumption that the first pulse makes all sites accessible to TTX. The envelope about the number of available channels during a train of pulses can, therefore, be approximated by a square function. The temporal behaviour of use dependence was limited by the binding kinetics of the TTX molecules. For the more rapidly bound STX deviations could occur because of 1) the frequency dependence observed (Fig. 1) and 2) the differences between τ_{on} and τ mentioned above. It can thus be, that the rapidly bound STX 'sees' the individual relaxing affinity increases per pulse.

Toxin charge

There are striking differences in the potential-dependence of use dependence between TTX and STX (Lönnendonker 1989). This dependence was almost identical at the toxin concentrations 4 and 8 n*M*. The nearly four times steeper increase in STX block is thus not a function of toxin concentration but could reflect the charge differences between the toxins. However, the voltage dependence of phasic toxin block is also affected by other factors: The frequency dependence of the STX actions (Fig. 1) can explain a part of the difference between TTX and STX because the more rapidly bound STX (Ulbricht and Wagner 1975; Wagner and Ulbricht 1975; Lönnendonker 1989) could bind to more channels than the slower TTX. Additionally, a saturation in decline ratio for TTX (not shown) was reached for frequencies below 2.0 Hz in contrast to 10 Hz in heart cells (Eickhorn et al. 1990). Thus the slopes of the regression lines are not only determined by the charge but also by the kinetics of the toxins.

Dependence of inactivation

A dependence of TTX block of Na^+ channels on inactivation was shown for cardiac cells (Carmeliet 1987; Eickhorn et al. 1990) and attributed to a higher toxin affinity of this state. Cohen et al. (1981) reported a slowing of recovery from inactivation of the TTX-blocked channels. Similar results have been reported for the use dependence of local anaesthestics and can be explained by assuming that these drugs bind more tightly to the inactivated form of the channel (compare Hille 1984; Strichartz 1973). Recently, Patton and Golding (1991) reported that use dependence of sodium channel IIA from rat developed at

pre-pulse potentials that do not elicit significant activation of the channels. My results show that (1) a prolonged hyperpolarization drives more channels into a state which favours use dependence and that (2) pulse amplitudes which can trigger inactivation of Na^+ channels but not activation produce the additional toxin block (Fig. 3). Thus these results demonstrate (1) a voltage dependence and (2) a use dependence possibly related to the resting and inactivated states of channels. Result (2) is especially difficult to reconcile with the observation that very short trigger pulses to $V_p = 60$ mV can also produce use-dependent effects (Lönnendonker 1989; Salgado et al. 1986). Moreover, in the region between $V_p = 0$ and 40 mV the time constants of inactivation approach the conditioning pulse time (Neumcke et al. 1987). Thus a pulse of 12 ms duration, as used in my experiments, can only drive between 50 to 70% of the channels into the inactivated state. Another problem is that the development of the use dependence is hidden under the 40 pulses to V_p. Thus the development may be slower at $V_p < 10$ mV, changing τ, or the proportion of channels which are influenced by V_p may be changed. In other words, it is not known what r_{use} represents and whether the strict correlation between r_{use} and h_∞ is influenced by the choice of this particular evaluation criterion.

Evidently, an activation of the channels was not necessary to elicit use dependence, and hence an escape of trapped ions inward (Salgado et al. 1986) is unlikely. This result and the cation dependence of additional toxin block (Lönnendonker 1991 b) imply that inactivation directly enhances the affinity of the binding sites for various cations and toxins. Such an interrelation between the internal inactivation gate and external cations was described before for Ni^{2+} (Conti et al. 1976) and for TTX and Zn^{2+} (Gilly and Armstrong 1982).

Inhibition of inactivation

The inactivation of Na^+ channels is easily impaired by chemical agents and enzymes, e.g. by chloramine-T (Wang 1984). Reports on the effects of guanidinium toxins on modified channels are sparse. Ulbricht et al. (1986) found differences in the on-kinetics of TTX block in myelinated nerve with $\tau_{on} = 39.6$ s after, compared to 26.4 s before treatment with chloramine-T, and the use-dependent block by TTX in heart cells was completely removed by batrachotoxin (Huang et al. 1987). Sodium channels with a 10-fold slower 'fast inactivation' had similar use-dependent characteristics to wild-type channels when expressed in oocytes (Patton and Goldin 1991). As shown in Figs. 4 and 5, fibres after treatment with chloramine-T have a modified use dependence. The rate of STX binding in these fibres was dependent on the holding potential, and the ratio of current decline was potential independent, in contrast to untreated fibres. However, it is not clear whether these results were produced by the inhibition of inactivation because chloramine-T could also alter other sites (Hahin 1990). Indeed the dependence of use-dependent TTX or STX block on external cations (Lönnendonker 1991 b) suggests that this effect is also modulated by external receptors which are linked to internal sites, e.g. to the inactivation gate.

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