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Binding of tetrodotoxin and saxitoxin to Na^+ channels at different holding potentials: fluctuation measurements in frog myelinated nerve

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The number of available Na^+ channels in nodes of frog nerve fibres was determined from nonstationary Na^+ current fluctuations recorded during a train of depolarizing test pulses. Mean numbers in Ringers's solution were 90000 at a hyperpolarizing holding potential $V_H = -40$ mV, 50000 at the resting potential ($V_H = 0$ mV) and 30000 at a depolarizing holding potential $V_H = 30$ mV. Addition of the cationic channel blockers tetrodotoxin (TTX) or saxitoxin (STX) to Ringer reduced the channel number by a factor which was independent of the holding potential. The reduction factor was 4 for 9.3 nM TTX and 3 for 3.5 nM STX. Thus, in the state of repetitive stimulation, TTX or STX blockage of Na^+ channels is hardly affected by the membrane potential. Taking into account use-dependent TTX and STX effects [1], it is concluded that binding of both toxins exhibits a weak voltage dependence with toxin affinities decreasing at more negative holding potentials. The results suggest that binding of TTX and STX occurs at an external superficial receptor near the Na^+ channel and that the toxin affinity of the receptor may be modulated by fast Na^+ activation and slow inactivation gating processes.

Introduction

This paper continues the analysis of binding of the neurotoxins tetrodotoxin (TTX) and saxitoxin (STX) to Na^+ channels in *Ranvier* nodes of frog myelinated nerve. In the preceding work [1], it was shown that repetitive stimulation increases the TTX or STX affinity of Na^+ channels at negative holding potentials (state B), relative to unstimulated channels (state A). Thus we have to differentiate between two states of toxin affinity at negative holding potentials.

The present investigation was performed to reveal the absolute affinity of Na^+ channels to these toxins at different holding potentials. This required the determination of the number of available nodal Na^+ channels with and without the presence of TTX or STX. These results cannot be found in Ref. 1 because (1) the necessary controls without toxin were not examined; and (2) only macroscopic currents were considered. However, the channel number cannot be taken as proportional to the macroscopic Na^+ current through all channels. An addition of a toxin or variation of the holding potential may not only change the number of available Na^+ channels but in addition the single-channel conductance and possibly the open-channel probability [2]. Therefore, non-stationary current fluctuations [3] were measured and analysed to obtain the channel number under various experimental conditions. The fluctuation analysis is based on small differences between currents elicited by a long train of identical test pulses. To limit the duration of the experiments, the pulses have to be applied at short intervals. Thus, the channel numbers derived from fluctuation measurements refer to the condition at which use-dependent toxin effects have already reached a stationary state (state B). I have found that under these conditions TTX and STX exhibit no detectable voltage-dependent binding to Na^+ channels within the range of holding potentials studied. The toxin binding to unstimulated fibres in state A has to be concluded by taking into account the results of the previous paper [1]. Therefore unstimulated Na^+ channels may have a lower affinity for TTX and STX at more negative holding potentials.

Part of the Results has been published before [4].

Abbreviations: TTX, tetrodotoxin; STX, saxitoxin.

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Methods

Na^+ currents were measured in nodes of frog motor nerve fibres at 15°C as described in the preceding paper [1]. The compositions of the extra- and intracellular solutions were also identical to those used in the previous investigation. In addition, an extracellular solution of low pH, but of otherwise identical composition, was used for rapid wash-out of TTX.

At the beginning of the experiment Na^+ currents at different test pulses, V (the potential scale V is defined with respect to the normal resting potential), were displayed on the oscilloscope and the reversal potential, V_0, of the currents was determined. All subsequent current measurements were performed under computer control. After the start of an 8 ms test pulse to $V = 60$ mV, the current was recorded at 10 μs intervals for the first 200 points and at 100 μs intervals for another 60 points. The currents were filtered through a four-pole low-pass Bessel filter with a cut-off frequency of 10 kHz. All test pulses were preceded by a 50 ms pulse to $V = -30$ mV to obtain for all holding potentials, V_H, the same initial value h_0 of the inactivation variable, h, at the onset of the test pulse. The pause between two test pulses was 1 s.

The measurements of non-stationary mean currents and current fluctuations were performed by an ensemble averaging technique [3]: From the onset of the kth pulse to the end, 260 current samples $I_k(t)$ were recorded. For each time t, the mean current values $I(t)$ and the corresponding variances, var(t), were calculated from a group of six subsequent records $I_k(t)$ according to:

$$I(t) = 1/6 \cdot \Sigma I_k(t) \tag{1}$$

$$\mathrm{var}(t) = 1/5 \cdot \Sigma (I_k(t) - I(t))^2 \tag{2}$$

This procedure minimizes errors introduced by possible slow drifts in the baseline current. Normally the calculation of $I(t)$ and var(t) was carried out on 15 groups each consisting of six records. The mean and variance values of the last 14 groups were averaged, stored and used for the subsequent analysis.

A virtually stationary level for use-dependent TTX and STX effects at hyperpolarizing holding potentials was reached by applying at least 40 test pulses in advance before recording the first current sample $I_1(t)$. This eliminates more than 80% of the maximal use-dependent effect for this stimulus frequency and for this negative holding potential V_H (compare current values at 50 s in Fig. 2 of Ref. 1).

Analysis

Ensemble average values of mean currents $I(t)$ and variances var(t) during depolarizing test pulses were processed as follows: Excess variances at the beginning of the pulse were discarded and isochronal (var, I) pairs were arranged in the sequence of growing absolute I values. The relation:

$$\mathrm{var} = i \cdot I - \frac{I^2}{N} + c \tag{3}$$

was then fitted to the data, where i is the current through one open channel, and N the number of available channels per node [3]. The variance, c, at $I = 0$ represents noise contributions not arising from Na^+ channels (background noise). This was shown in control experiments with alternating positive and negative control pulses. Here, the parameter c assumed much smaller values while the fitted values of i and N remained virtually unchanged. Hence, the determination of the channel current and the channel number is not affected by the background noise. From i the chord conductance:

$$\gamma = i/(V - V_0) \tag{4}$$

of a single Na^+ channel was calculated.

Different values of mean currents were affected differently by series-resistance artifacts. To exclude this from the results the channel number, N, was corrected by a procedure described in Ref. 5. In short, the corrected values N_c were derived from:

$$N_c = N/((1 - R_s \cdot N \cdot i)/(V - V_0)) \tag{5}$$

where R_s denotes the resistance in series with the nodal membrane, which was assumed to be 220 kΩ for frog nodes [6]. Correcting the channel number with Eqn. 5 also takes into account the varying impedance of the nodal membrane during the transient Na^+ current [5].

Fitting

The fit of the var(I) data by Eqn. 3 and the determination of linear regressions of the channel numbers, N_c, were done by conventional least-square methods. The 95% confidence limits were calculated by methods found in Ref. 7.

Results

Fluctuation measurements in toxin-free solutions

Fig. 1 illustrates the results of fluctuation measurements which were performed on a single fibre held at a holding potential V_H of 20 mV and later at −40 mV. Part A of the figure shows the mean currents $I(t)$ during the pulse at both holding potentials and part B the var(t) relation for $V_H = -40$ mV. The isochronal var(I) data for the same holding potential are plotted in part D together with the fit by Eqn. 3. The corresponding fitted curve for $V_H = 20$ mV in part C has a differ-

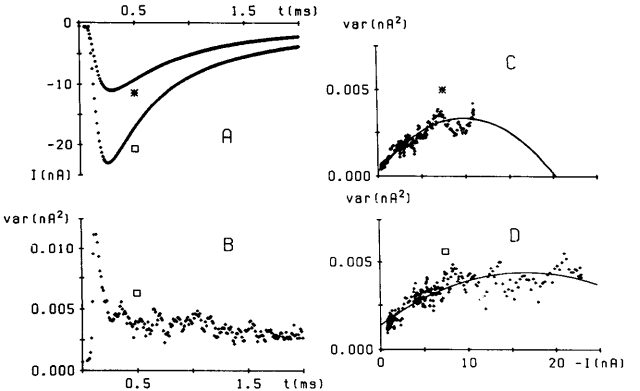

Fig. 1. Na^+ currents (A) and variances (B) vs. time, and variances vs. current (C and D) at two different holding potentials. Currents and variances were recorded without toxin at $V_H = 20$ mV (*) and -40 mV (□). In A and B only the first 2 ms of the currents and variances are shown.

ent curvature owing to the reduced number of available Na^+ channels at more positive holding potentials. The channel numbers N_c are 111 000 and 40 000, the corresponding channel conductances γ are 5.75 and 9.14 pS at $V_H = -40$ and 20 mV, respectively.

The results of this single experiment exhibit a general trend which was also found in other fibres: larger macroscopic Na^+ currents during pulses at more negative holding potentials are produced by a higher number N_c of available Na^+ channels which, however, have a reduced single-channel conductance γ. Mean N_c values for different holding potentials from several experiments in toxin-free solutions are shown in Fig. 2A and the negative correlation between N_c and γ in Fig. 5 (symbols * in part B).

N_c at different V_H without and with TTX

In toxin-free solutions the dependence of the channel number N_c on the holding potential V_H seems to be a linear relation, but the scatter of the means is considerable (Fig. 2A). Reasons for this great variability in the number of available Na^+ channels per node are: (1) intrinsic error sources of the fluctuation analysis owing to nonstationary conditions during long current measurements. (2) Variation of the channel number between different fibres, e.g., higher numbers per node at larger fibre diameters. (3) Seasonal variations of channel numbers, e.g., high numbers for frogs delivered in autumn and steadily decreasing numbers during the following months of captivity (Fig. 2C).

In the presence of 9.3 nM TTX, a comparable scatter of the mean channel numbers but again the same linear relation between N_c and V_H is observed to that in toxin-free solutions (Fig. 2B). This is illustrated with the regression line and the 95% confidence interval in Fig.

TABLE I

Ratios ± S.E. of Na^+ channel parameters without and with toxin in the extracellular solutions as determined from measurements at the same fibre

N_c, corrected number of available channels; γ, single-channel conductance; I_p, peak Na^+ current at $V = 60$ mV. Number of experiments in brackets. The protocols I and II are described in Results. A ratio of >1 means a decrease in the value in toxin solutions, <1 means an increase.

Protocol	V_H (mV)	toxin (nM)	ratio N_c	ratio γ	ratio I_p
I	20	9.3 TTX	4.00 ± 0.38 (4)	0.82 ± 0.02 (4)	3.23 ± 0.37 (5)
	−40	9.3 TTX	5.04 ± 0.93 (5)	0.92 ± 0.21 (5)	3.54 ± 0.16 (5)
II	20	9.3 TTX	4.31 ± 0.92 (4)	0.89 ± 0.14 (4)	2.47 ± 0.33 (4)
	−40	9.3 TTX	3.81 ± 1.21 (4)	0.90 ± 0.11 (4)	2.76 ± 0.33 (4)
I	20	3.5 STX	2.79 ± 0.45 (5)	0.87 ± 0.18 (6)	3.21 ± 0.23 (8)
	0	3.5 STX	2.65 ± 0.20 (9)	0.82 ± 0.06 (12)	4.11 ± 0.25 (12)
	−40	3.5 STX	3.13 ± 0.82 (6)	0.66 ± 0.07 (8)	4.81 ± 0.22 (8)

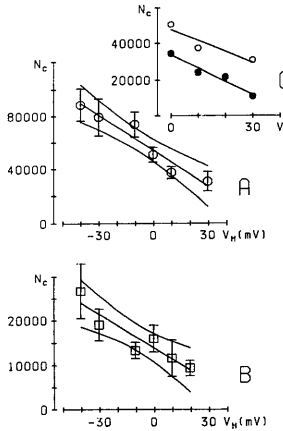

Fig. 2. Channel number N_c vs. holding potential V_H in extracellular solutions without (A) and with 9.3 nM TTX (B). The symbols (in A and B) denote mean values from at least seven fibres and the bars the S.E.M. values. The regression lines (in A and B) are shown together with their 95% confidence limits. The ordinate in (B) is scaled down from (A) by the factor 4. In (C) the values of (A; ○) for V_H between 0 and 30 mV are compared with values from 12 months elder frogs (●).

2B (the ordinate is scaled down by a factor 4 in comparison with Fig. 2A).

To avoid the uncertainties introduced by the large variation of channel numbers in different experiments, ratios of channel parameters without and in the presence of toxins were determined from fluctuation measurements in single fibres. Two different types of experiment were used to determine the ratios at $V_H = -40$ mV and 20 mV without and with 9.3 nM TTX:

Protocol I: Current fluctuations were first recorded at both holding potentials without toxin and, thereafter, at the same holding potentials in the presence of TTX. Illustrative examples with 9.3 nM TTX at the holding potentials $V_H = -40$ and 20 mV are shown in Fig. 3. The N_c values from this experiment are in the range of the mean values from Fig. 2B, but the peak Na^+ current $I_p = -2.1$ nA at $V_H = -40$ mV is considerably smaller than the mean. Ratios of channel numbers N_c, channel conductances γ and peak Na^+ currents I_p at $V = 60$ mV obtained with this protocol are compiled in the first rows of Table I. In agreement with Fig. 2 no clear voltage-dependence of TTX binding to Na^+ channels was found from these fluctuation measurements (N_c ratios at $V_H = -40$ and 20 mV are not significantly different).

A disadvantage of protocol I is that the TTX results were always obtained at the end of an experiment and this could introduce a bias into the analysis. Therefore, fluctuation experiments were also performed in a different order (*Protocol II*): Current fluctuations were first recorded in the presence of TTX at both holding potentials. The external solution was then exchanged against a toxin-free solution with a pH of 4.5 to accelerate wash-out of TTX [12]. This solution was applied for 10 min and subsequently replaced by Ringer. After a pause of 10 min fluctuation measurements at $V_H = -40$ and 20 mV were performed again. The results obtained with protocol II are listed in the third and fourth rows of Table I. Again, no significant voltage-dependence of the TTX affinity can be deduced from the N_c ratios at both holding potentials. The relative peak Na^+ currents I_p obtained with protocol II are smaller than those obtained from protocol I. This could be caused by incomplete wash-out of TTX in the experiments with protocol II.

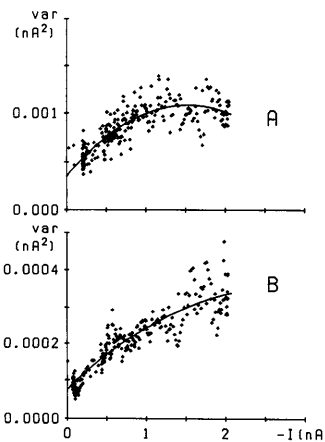

Fig. 3. Variances vs. current at the holding potentials $V_H = 20$ mV (A) and -40 mV (B) in the presence of 9.3 nM TTX. The data are from two fibres with channel numbers near the mean of the examined fibre population. The corrected channel numbers N_c are 3300 (A) and 23200 (B).

N_c at different V_H without and with STX

The effects of 3.5 nM STX on nodal Na^+ channels were determined with protocol I at three different holding potentials V_H of −40, 0 and 20 mV. Fig. 4 shows examples of var(I) data obtained from a single fibre, the parameters of this particular experiment are listed in the legend. Channel parameters in 3.5 nM STX (referred to toxin-free solution) from this and more fibres are compiled in the last three rows of Table I. As in the TTX experiments, there is no clear dependence of the STX-induced reduction of channel number N_c on holding potentials V_H between 20 and −40 mV. Thus the observed increase of the I_p ratios at more negative holding potentials is entirely due to a concommitant decline of the ratio of the channel conductances γ (compare Fig. 5), which is here more pronounced than in the experiments presented above.

Dependence of γ on channel number N_c

The single-channel conductance, γ, is negatively correlated with the number of available channels N_c. Fig. 5A shows this correlation for the experiments in the presence of TTX or STX (the same ○ symbols for both types of toxins), and in Fig. 5B all results with and without toxin are combined. Both figures contain only values from experiments in which the axoplasm resistance could be measured to allow the determination of absolute conductance values. The regression lines in part A and B are not different within the confidence limits. Despite the large scatter of the data, the regression analysis reveals that an increase by 10000 open channels decreases the single-channel conductance, γ, by 0.86 pS. Owing to the large scatter of the data in Fig. 5A and B, types of correlation between N_c and γ other than linear were not investigated.

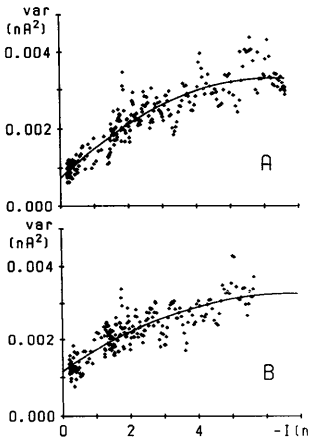

Fig. 4. Variances vs. current at the holding potentials $V_H = 0$ (A) and −40 mV (B) from one fibre with 3.5 nM STX in the extracellular solution. The corrected channel numbers N_c are 16800 (A) and 53500 (B).

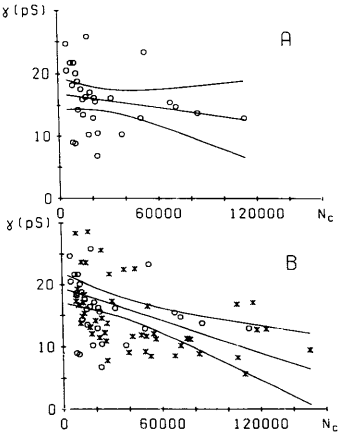

Fig. 5. Single-channel conductance, γ, vs. channel number, N_c, for all available data from experiments with STX or TTX in the extracellular solution (A: ○) and combined with data without toxin (B; •). Shown are the regression lines with 95% confidence limits. The corresponding regression lines are:

$$\gamma = -0.36 \cdot 10^{-4}\,[\pm 0.69 \cdot 10^{-4}] \cdot N_c + 17.0\,[\pm 2.6] \text{ for (A)}$$

and

$$\gamma = -0.86 \cdot 10^{-4}\,[\pm 0.48 \cdot 10^{-4}] \cdot N_c + 19.0\,[\pm 2.5] \text{ for (B)}$$

In brackets the 95% confidence limits for slopes and ordinate intercepts.

Discussion

Validity of the fluctuation analysis

To study possible effects of holding potentials on TTX or STX binding to Na^+ channels, preparations with a large channel number must be used to allow the detection of small variations in these numbers. *Ranvier*

nodes of myelinated nerve contain thousands of Na^+ channels and are, therefore, suitable to perform these investigations. A disadvantage of such a multi-channel preparation is that the number of available ionic channels cannot be directly counted but must be calculated indirectly from fluctuations of the ionic currents. The assumptions used in the analysis of nonstationary current fluctuations are [3]: (i) the channels have only two conductance states (closed–open); (ii) all channels are identical; and (iii) neighbouring channels do not interact with each other. Though deviations from these assumed properties have been reported for Na^+ channels in various tissues [8–10], the majority of the channels seem to fulfil the requirements needed to apply Eqn. 3 to nonstationary current and variance values. Furthermore it was shown that i values from single channel and variance analysis are very similar in the same preparation [11].

Negative correlation between conductance, γ, and channel number, N_c

Addition of TTX or STX to the extracellular solution reduces the number N_c of available Na^+ channels in the node of *Ranvier*, and increases the single-channel conductance, γ (Table I). The negative correlation between γ and N_c is also observed in toxin-free solutions and shows no difference between the monovalent TTX and the divalent STX (Fig. 5A and B). This suggests that the increase of γ at reduced channel numbers is not caused by the presence or the charge of the toxin molecule. Instead, interactions between neighbouring open Na^+ channels could affect the single-channel conductance, e.g., by depletion of Na^+ ions in the unstirred solution layers around the external channel mouth [2].

TTX and STX binding to unmodified Na^+ channels

The fluctuation experiments described in this paper have not revealed a clear voltage-dependence of TTX or STX binding to Na^+ channels for holding potentials, V_H, between −40 and 20 mV (Table I, and Fig. 2). In other words, it seems that TTX and STX bind to stimulated Na^+ channels nearly independently of the holding potential. As explained in the Introduction, this result refers to Na^+ channels with fully developed use-dependent toxin effects (channels in state B). But it was shown in Ref. 1 that the Na^+ current also decreases during repetitive pulsing at negative holding potentials. After more than 40 pulses it reaches the new steady-state level, which was examined here. Such a decline of Na^+ current must be the consequence of an increasing affinity for the toxins during stimulation. Since the use-dependent decline of Na^+ currents in the presence of TTX or STX is more pronounced at $V_H = -40$ mV compared to 20 mV [1], unstimulated Na^+ channels (state A) tend to have a lower toxin affinity at more negative holding potentials. However, the voltage-dependence of toxin binding is probably rather small. As an example, take the absence of use-dependent effects in the presence of 3.5 nM STX at $V_H = 0$ mV and the 35% decline of macroscopic Na^+ currents during repetitive test pulses at $V_H = -40$ mV ([1]: Fig. 1). If this is interpreted by voltage-independent binding to stimulated Na^+ channels (state B), the STX equilibrium dissociation constant, K_d, of unstimulated channels (state A) changes maximally e-fold per 40 mV, and it increases with more negative holding potentials.

The absence of a pronounced voltage-dependence in the binding of TTX and STX to Na^+ channels in myelinated nerve is in agreement with two earlier investigations analysing macroscopic currents. Thus Ulbricht and Wagner [12] found the same TTX affinities in extracellular solutions of pH 7.2 within a 40 mV range of holding potentials. Also, an apparent voltage-dependence of TTX affinity at pH 5 could be interpreted by competition between H^+ and TTX without the need to invoke voltage-dependent toxin binding to Na^+ channels. Similarly, Rando and Strichartz [13] have concluded that STX inhibition of Na^+ currents is independent of membrane potential. These findings for myelinated nerve may not be applicable to Na^+ channels in other preparations. For example, Salgado et al. [14] suggested that STX block of Na^+ channels in crayfish giant axons is both voltage- and use-dependent and Cohen et al. [15] detected a pronounced voltage-dependence of TTX block for cardiac Na^+ channels.

TTX and STX binding to modified Na^+ channels

Na^+ channels modified by batrachotoxin (BTX) have TTX and STX equilibrium dissociation constants, K_d values, in the nM concentration range as unmodified channels. However, toxin binding to these modified channels is strongly dependent on membrane potential. This has been shown for the first time for BTX-modified Na^+ channels in lipid bilayer membranes [16] and subsequently also for myelinated nerve [17]. As an example, the K_d value of STX binding to BTX-modified Na^+ channels in lipid bilayers changes e-fold per 40 mV [18,19] but it decreases with more negative potentials, opposite to my results. Thus, the type of voltage-dependence found in BTX-modified channels is not found in unmodified myelinated nerve fibres.

Voltage-dependent TTX and STX binding has only been observed for Na^+ channels modified by BTX, but not for channels treated with chloramine-T [20]. A difference between these gating modifiers is that BTX affects both the activation and inactivation of Na^+ channels, whereas chloramine-T slows inactivation selectively [21,22]. This suggests that the voltage-dependent TTX and STX binding to BTX-modified channels is related to alterations in the activation process.

Models of TTX or STX blockage of Na^+ channels

The blockage of Na^+ channels by TTX and STX has been explained using two different models. One was proposed by Kao and Nishiyama [23] and further elaborated by Hille [24]. In this model a toxin molecule physically blocks the passage of other ions by binding to the external selectivity filter of the channel and thus acts like a 'cork in a bottle'. However, more recent work showed that changes in toxin affinity and changes in ionic selectivity are not correlated as expected if the toxin binds to the selectivity filter (reviewed in Ref. 25). Another difficulty with the 'plug' model is the absence of a pronounced voltage-dependence in the TTX and STX binding to unmodified, stimulated Na^+ channels (state B). Furthermore, the toxin affinity in unstimulated fibres (state A) which tend to decrease with hyperpolarization and the similar behaviour of the monovalent TTX and the divalent STX are not compatible with this model. The results with BTX-modified channels show voltage-dependence of block increasing at negative holding potentials, but the univalent TTX and the divalent STX have similar voltage-dependencies [18,19] and even this excludes a binding site in the membrane electric field.

In the second model of Kao and Walker [26], the TTX and STX receptor is postulated to be at the external membrane surface outside of the channel, and bound toxin molecules are thought to obstruct the channel more like a 'lid' than a 'plug'. This model can account for the observed TTX or STX block of unstimulated Na^+ channels which is weakened at more negative holding potentials and thus shows a voltage-dependence opposite to that expected from the 'plug' model. Such behaviour can be interpreted with voltage-dependent conformational changes of an external toxin receptor yielding a lower affinity for TTX and STX at more negative membrane potentials.

Use- and voltage-dependent TTX and STX effects

The results presented in this and the previous paper [1] show that TTX and STX blockage of nodal Na^+ channels depends on the frequency of test pulses as well as on the holding potential. This complex behaviour makes it difficult to discriminate clearly between use- and voltage-dependent toxin effects, and it requires the distinction between TTX and STX affinities for unstimulated (state A) and stimulated (state B) Na^+ channels. As shown above, both types or states of Na^+ channels exhibit no or only a weak voltage-dependence of TTX and STX binding which is compatible with a superficial toxin receptor near the Na^+ channel. To account for the use-dependent TTX and STX effects, this receptor should assume two conformational states, A and B, with low and high toxin affinities, respectively. Holding the fibre at negative potentials favours the low-affinity state A, whereas a short positive test pulse triggers a transition to the high-affinity state B with a subsequent decline of the Na^+ current. At positive holding potentials there are no use-dependent TTX or STX effects, hence both states A and B merge into one stimulus-independent receptor state. Thus the affinity of the TTX and STX binding site depends both on short and on long-lasting changes of the membrane potential. This suggests that the affinity of the TTX and STX receptor may be modulated by fast Na^+ activation as well as by slow Na^+ inactivation gating processes.

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