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Use-dependent block of sodium channels in frog myelinated nerve by tetrodotoxin and saxitoxin at negative holding potentials

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Na^+ currents were measured in myelinated frog nerve fibres in the presence of nanomolar concentrations of tetrodotoxin (TTX) or saxitoxin (STX) in the extracellular solution. The Na^+ currents declined during a train of depolarizing pulses if the fibre was held at hyperpolarizing potentials between the pulses. At a pulse frequency of 0.8 Hz, the peak Na^+ currents were reduced to 70 or 60% of the initial value in 9.3 nM TTX and 3.5 nM STX solutions, respectively. A decline of Na^+ currents was also observed in two-pulse experiments. The peak Na^+ current during a second test pulse did not depend on the duration (0.2 to 12 ms) of the first pulse. It decreased with increasing interval between the pulses, reached a minimum and increased again. The results are interpreted with a use-dependent blockage of Na^+ channels by TTX or STX at negative holding potentials. The effects were described quantitatively, assuming a fast affinity increase of toxin receptors at Na^+ channels triggered by Na^+ activation followed by slow toxin binding to channels and relaxation of the receptor affinity.

Introduction

The term 'use-dependence' was introduced by Courtney [1] to describe the cumulative effects of local anaesthetics during repetitive stimulation. In his experiments on myelinated nerve fibres, a train of depolarizing pulses elicited smaller and smaller Na^+ currents in the presence of a quaternary derivative of lidocaine in the axoplasm. As an interpretation of the results it was suggested that charged forms of local anaesthetics can reach an inner blocking site in the Na^+ channel only from the internal solution and only when the channel gate is open [2]. Subsequently, a use-dependent block of Na^+ channels was also found for tetrodotoxin (TTX) and saxitoxin (STX) which bind to outer channel sites. These toxins decrease the Na^+ currents during repetitive pulses in heart cells [3–5] and in developing rat muscles [6]. On the other hand, no such effects were found in myelinated nerve fibres held at the normal resting potential [7].

The different effects of TTX and STX on Na^+ channels in various membranes might be correlated with different equilibrium dissociation constants of these toxins. They are in the nanomolar concentration range for axon membranes but of the order of several μM for heart and embryonic muscle [3,6]. However, it is not possible to discriminate strictly between Na^+ channels with high and stimulus-independent TTX and STX affinities and channels with lower toxin affinities and use-dependent block. Thus, experiments on crayfish giant axons containing high-affinity receptors for TTX and STX have revealed a decrease of Na^+ currents in the presence of the toxins upon repetitive stimulation [8]. However, in this preparation the use-dependent effects of TTX and STX were only observed at negative holding potentials but not at the normal resting potential.

In experiments on *Ranvier* nodes of frog nerve I have obtained similar results which confirm and extend the observations reported for crayfish axons. In particular, I have studied the effects of holding potential and of various pre- and test pulses on the development and recovery of use-dependent TTX and STX effects at different toxin concentrations. The time course of the use-dependent effects could be quantitatively described, assuming slow intrinsic membrane processes altering the toxin affinity of channel receptors, and taking into

Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; Mops, morpholinepropanesulphonic acid.

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account the extrinsic on- and off-rate constants of toxin binding to these receptors. A general conclusion from the results is that a short opening of the channel gate on the inner membrane side can exert a long-lasting modification of the external toxin receptor. Hence, TTX or STX binding and gating of Na^+ channels can no longer be considered to be independent processes.

Part of these results has been published as abstract [9].

Methods

Single motor fibres were dissected from the tibial nerve of the frog *Rana esculenta* as described by Stämpfli and Hille [10]. The fibres were mounted in a nerve chamber, and the node in the central compartment was continuously perfused by extracellular solutions at 15°C and voltage clamped [11]. K^+ currents were blocked by intracellular application of CsCl and extracellular tetraethylammonium chloride (TEA · Cl). At the beginning of the experiment the membrane potential was adjusted to obtain a steady-state Na^+ inactivation equivalent to $h_\infty = 0.7$. This potential was taken as the resting potential of amphibian myelinated nerve fibres (−71 mV, see Ref. 12), and deviations from this potential are denoted by V. Effects of the resistance, R_s, in series with the nodal membrane on the actual membrane potential were estimated to be smaller than 3 mV (peak Na^+ current < 10 nA, $R_s = 220$ kΩ, see Ref. 13) and therefore neglected. The majority of the linear components of leakage and capacity currents were compensated by an analogue circuit. At the end of the experiments, the axoplasm resistances between the node and the cut ends of the fibre were measured electrically to allow calibration of the Na^+ current [14].

Solutions

The external solution contained 110 mM NaCl, 2 mM $CaCl_2$, 10 mM TEA · Cl, 4 mM Mops and the pH was adjusted to 7.2 (18°C) with NaOH. To this solution different amounts of tetrodotoxin (TTX, Sigma) or saxitoxin (STX, a gift from Professor J.M. Ritchie, Yale University) were added. The internal bath solution contained 113 mM CsCl, 7 mM NaCl, 4 mM Mops at a pH of 7.2 (18°C).

Measurements

The setting of the holding potential, the application of pre- and test pulses and the recording of Na^+ currents were done under computer control (LSI 11/24: the hardware components and technical details are described in Ref. 15). After the onset of the test pulses, the currents were sampled at 10 μs intervals for the first 200 data points and at 100 μs intervals for 100 more points until the end of the pulses. The currents were filtered through a four-pole low-pass Bessel filter with a cut-off frequency of 10 kHz and stored on a winchester. The computer program allowed to set prepulses of variable duration and to select different pauses between pre- and test pulses which were always to $V = 60$ mV. Details of the pulse protocols are described in Results. Negative pulses for on-line subtraction of capacity and leakage currents were not applied to avoid possible interferences with use-dependent effects.

Analysis

Peak Na^+ currents, I, were determined under visual inspection of the currents. The peak current which is not influenced by use-dependent effects (the control) is denoted by I_0 and all other peak currents after the time, t, by I_t. In the repetitive pulse experiments peak currents, I_t, denote means of a group of several subsequent peak current values (compare legend to Fig. 2). As time of the mean value, the time of the last pulse in the group was chosen.

Part of the experimental data were fitted by the method of least squares [16]. The formula had the form:

$$I_t/I_0 = 1 - c \cdot (e^{-t/\tau_{on}} - e^{-t/\tau_{off}}) \quad (1)$$

for two-pulse experiments. In this equation, t means the time from the onset of the use-dependent effect, c is a fitted constant between 0 and 1, and τ_{on} and τ_{off} denote time constants of the on and off responses.

Experiments with repetitive pulsing were fitted with one exponential (I_∞ denotes the steady-state value of the peak current, and τ is a time constant):

$$I_t/I_0 = (1 - I_\infty/I_0) \cdot e^{-t/\tau} + I_\infty/I_0 \quad (2)$$

Results

Effects of repetitive test pulses

Fig. 1 illustrates Na^+ currents at $V = 60$ mV in the presence of 9.3 nM TTX or 3.5 nM STX in the extracellular solution. For each solution, measurements were performed at two holding potentials of $V_H = 0$ and −40 mV. After a change of the holding potential 8–10 min were waited to establish a new stationary state. Test pulses were applied at a frequency of 0.8 Hz and means of subsequent Na^+ current traces are shown in the Fig. 1. It is evident from Fig. 1 that the currents decreased during repetitive pulsing at the holding potential $V_H = -40$ mV, but not or only slightly at $V_H = 0$ mV. In no case, was the time course of Na^+ activation or inactivation altered during repetitive pulsing. This suggests that the test pulses affected all Na^+ channels equally and not only a specific channel population. The observed decline of Na^+ currents at negative holding potentials is caused by the toxins TTX or STX

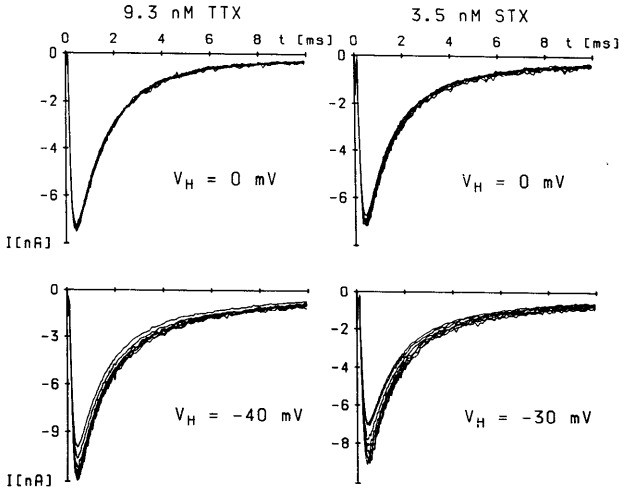

Fig. 1. Na^+ currents during a train of depolarizing test pulses. Currents were recorded in 9.3 nM TTX (left) and 3.5 nM STX (right) solutions at 15 °C and at the holding potentials $V_H = 0$ mV and -40 or -30 mV. Test pulses were applied to $V = 60$ mV at a frequency of 0.8 Hz. V_H and V denote deviations from the resting potential (see Methods). Shown are mean currents after 2.5 (2), 7.5 (2), 15.0 (3), 22.5 (3), 37.5 (4), 71.25 (7) and 126.25 s (10), the numbers in brackets denote the numbers of averaged subsequent current traces (see Methods).

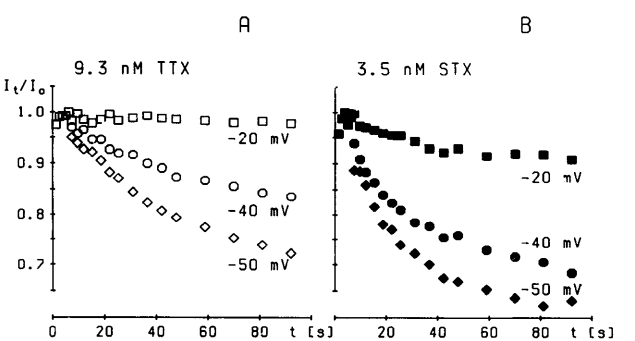

Fig. 2. Time course of normalized peak Na^+ currents during repetitive test pulses. Pulses were applied to $V = 60$ mV at a frequency of 0.8 Hz. Shown are normalized peak currents in 9.3 nM TTX (A) and 3.5 nM STX (B) solutions at the holding potentials $V_H = -20$, -40 and -50 mV (from above). The peak currents are means from 2 to 10 subsequent current traces.

because in control experiments at $V_H = -40$ mV with toxin-free solutions no effects of repetitive pulsing on Na^+ currents could be detected (not shown).

The use-dependent effects illustrated in Fig. 1 occur within some seconds during a train of depolarizing test pulses and have to be distinguished from the spontaneous decrease of Na^+ currents during long experiments. Such a decline has a time constant of about 30 min and occurs at all holding potentials and also without stimulation. To exclude such slow run-down effects from the following analysis all peak amplitudes were normalized with respect to the control value I_0.

Dependence on holding potential

Fig. 2 shows that normalized peak amplitudes in the presence of 9.3 nM TTX and 3.5 nM STX decrease during repetitive pulsing and that this effect becomes more pronounced at more negative holding potentials. Thus, at $V_H = -50$ mV the peak amplitudes were reduced to 70 and 60% of the initial value, I_0, in TTX and STX solutions, respectively. It can be seen that the currents in TTX (and probably also in STX) had not

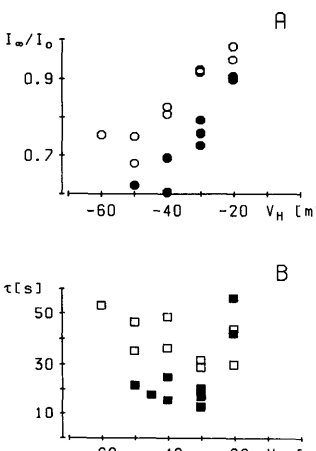

Fig. 3. Parameters determined by exponential fits of the decline of peak Na^+ currents as shown in Fig. 2. (A) Normalized stationary currents I_∞/I_0 for 9.3 nM TTX (open symbols) and 3.5 nM STX (closed symbols) as function of the holding potential V_H. (B) Time constants, τ, of the decline of peak Na^+ currents for 9.3 nM TTX (open symbols) and 3.5 nM STX (closed symbols) as function of the holding potential V_H.

TABLE I

Fit parameters of Eqn. 1 for the data of Fig. 4 and model parameters (see Appendix) for the calculated curves

The fit parameters are explained in the Methods. The k_{off} values are from [18,19]. Δk_{on} is the increase of k_{on} and τ^* the relaxation time constant of Δk_{on}.

Toxin (nM)	Fit parameters			Model parameters			
	τ_{on} (s)	τ_{off} (s)	c	k_{on} (10^6 M^{-1} $\cdot s^{-1}$)	Δk_{on} (10^6 M^{-1} $\cdot s^{-1}$)	k_{off} (10^{-2} s^{-1})	τ^* (s)
14.0 TTX	20.16	27.27	0.29	2.79	0.25	1.4	32.0
23.4 TTX	12.95	24.16	0.28	2.55	0.45	1.4	24.0
35.1 TTX	10.02	19.39	0.52	2.23	0.77	1.4	18.5
1.9 STX	1.25	42.32	0.05	2.00	1.5	1.8	4.0
3.5 STX	3.52	24.62	0.06	1.01	0.5	1.8	4.0
7.0 STX	2.83	7.68	0.48	2.90	1.3	1.8	4.0

reached a steady level I_∞ after 110 pulses to 60 mV applied at a frequency of 0.8 Hz.

To evaluate this current decline in more detail, the normalized steady-state value I_∞/I_0 was extrapolated from the fits of all repetitive pulse experiments with Eqn. 2. These values are shown in Fig. 3A as function of the holding potential. In agreement with Figs. 1 and 2, the I_∞/I_0 values are smaller in 3.5 nM STX (filled symbols) than in 9.3 nM TTX (open symbols). The time constant, τ, of the fitted function (2) shows no clear dependence on the holding potential or on the type of toxin (Fig. 3B). This suggests that at more negative holding potentials the number of channels available for the extra block during a pulse is increased, rather than the rate of TTX or STX binding during the interpulse interval (see section: Quantitative description of use-dependence).

Dependence on toxin concentration

Use-dependent TTX and STX-effects were so pronounced that two pulses, separated by a short interval Δt, were sufficient to demonstrate it. Owing to the use-dependence, the second pulse elicited a smaller current (amplitude I_t) than the first pulse (amplitude I_0). The two-pulse method was used to study the influence of different toxin concentrations on the development of the extra block. The gap time Δt was varied at a holding potential V_H of -40 mV. The first pulse elicited a peak Na^+ current (I_0) and, owing to the use-dependent effect, the second pulse a smaller peak amplitude (I_t). In Fig. 4 the normalized peak current I_t/I_0 is plotted against the gap duration Δt for three TTX and STX concentrations. The means of I_t/I_0 (from four to eight experiments) were fitted with the sum of a falling and a rising exponential function (Eqn. 1, see Methods). The experimental data and the fitted curves reveal that the gap time for maximum current depression decreases with higher toxin concentrations. In the presence of

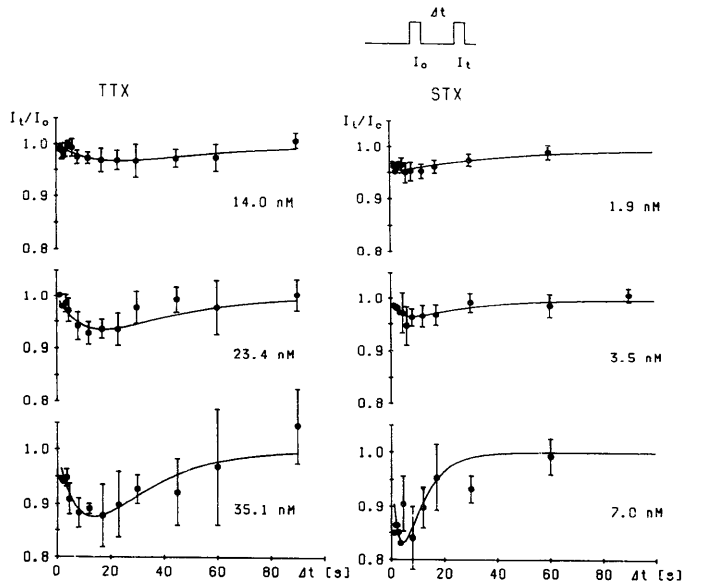

Fig. 4. Two-pulse experimeats with variable gap durations Δt (see pulse program as inset) at three TTX (left) and three STX concentrations (right). Holding potential $V_H = -40$ mV, pulses to $V = 60$ mV. Measurements with different gap times, Δt, were separated by a 2 min pause. Points of the shortest and longest gaps were studied alternatingly. Shown are normalized mean peak Na^+ currents of 4 to 8 two-pulse experiments and their S.D. values (S.D. not given for the shortest gaps). The peak current at time t is normalized with respect to the peak current at time zero. The curves are calculated with Eqn. 1 with the parameters given in Table I.

STX, the minimum of I_t/I_0 is reached earlier and at a lower toxin concentration than with TTX. As shown in Table I, the time constants τ_{on} and τ_{off} decrease with increasing toxin concentration; the least pronounced is the decrease of τ_{off} with increasing TTX concentration.

Effects of duration of the first pulse

To determine the minimum pulse duration needed to elicit use-dependent TTX and STX effects, two-pulse experiments were performed with a first pulse of varying duration and a constant gap time between the first and the second pulse. The results shown in Fig. 5 exhibit no dependence of I_t/I_0 on the duration Δt of the first pulse between 0.2 and 12 ms. Hence, the use-dependent effects of STX are already fully developed at 0.2 ms, i.e., at a time which is even shorter than the time to reach the peak Na^+ current (see Fig. 1).

Quantitative description of use-dependence

The experimental results on use-dependent blockage of Na^+ channels by TTX and STX show that the effects are triggered by short depolarizations but develop and relax rather slowly. These findings suggest a rapid affinity change of the toxin receptor during a single test pulse followed by slower toxin binding and release processes. In the upper part of Fig. 6, this complex toxin–receptor interaction is illustrated and the time constants for binding and unbinding as well as those for an affinity increase and decrease are indicated. For a quantiative description of the phenomena it will be

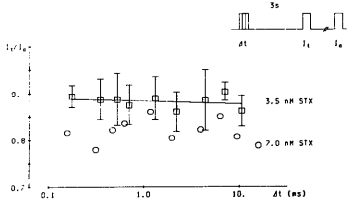

Fig. 5. Two-pulse experiments with a constant gap of 3 s and variable durations Δt of the first pulse (see pulse program as inset). The peak current I_t after 3 s is normalized with respect to the peak current I_0 measured 2 min later. This current is also used as control peak amplitude for the next two-pulse experiment. Shown are normalized peak Na^+ currents as function of the duration of the first pulse for 3.5 nM STX (mean and S.D. for four experiments) and for 7.0 nM STX (values from one experiment). Note the logarithmic scaling of the abscissa. The regression line for the 3.5 nM STX values shows that the means are hardly dependent on the duration of the first pulse.

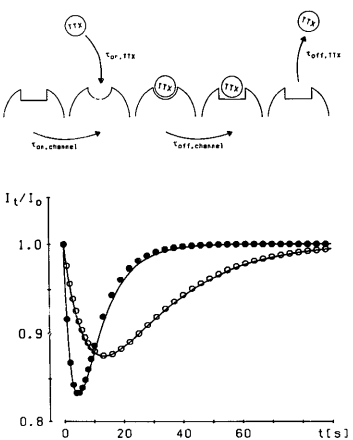

Fig. 6. (Upper part) A simple model of affinity changes of toxin receptor and toxin-binding. The intrinsic time constants for the receptor ($\tau_{on,channel} \ll \tau_{off,channel} = \tau^*$) and the extrinsic time constants of the toxin binding ($\tau_{on,TTX}$, $\tau_{off,TTX}$) are indicated. (Lower part) Model calculations for the two-pulse experiments of Fig. 4. Shown are values from the fit curves of Fig. 4 for the highest TTX (open symbols) and STX (filled symbols) concentrations and the model calculations as smooth curves. Table I contains the Δk_{on} and k_{off} values of the curves.

assumed that the affinity change triggered by a single pulse relaxes with a time constant τ^* and that the occupancy to toxin receptors R by toxin molecules T is governed by the first-order reaction:

$$T + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} TR \qquad (3)$$

with forward and backward rate constants k_{on} and k_{off}. The definition of the other model parameters and details of the calculations can be found in the Appendix.

A comparison between the fitted curves describing the data of Fig. 4 for the highest TTX and STX concentrations and normalized peak Na^+ currents computed from the model is presented in the lower part of Fig. 6. Values of the used model parameters for all TTX and STX concentrations can be found in Table I.

Discussion

Interpretation of use-dependent TTX and STX effects

Previously, use-dependent blockage of Na^+ channels by TTX or STX was explained with two different hypotheses. It was either assumed that the rate constants of toxin binding depend on the gating state of the channel [4,5] or that they are affected by Na^+ or Ca^{2+} ions trapped in the channel [8]. For the ion-trapping mechanism it was postulated that monovalent permeant Na^+ ions and divalent impermeant Ca^{2+} ions in the channel exert the same effects on the binding of the cationic toxins TTX or STX. Since this seems to be unlikely, I favour the idea of state-dependent toxin affinities, which is extended to include slow toxin binding to and unbinding from the channel receptor.

The results presented in Fig. 5 show that 0.2 ms conditioning pulses to $V = 60$ mV are sufficient for a maximal use-dependent block and that longer pulses do not produce a larger effect. The corresponding time constants τ_m and τ_h of Na^+ activation and of the early phase of Na^+ inactivation at the same potential and at the same temperature of 15°C are $\tau_m = 0.085$ ms and $\tau_h = 0.69$ ms [17]. This suggests that the affinity change of the toxin receptor is triggered by a short activation of the Na^+ channel whereas the subsequent inactivation does not give a further modification. An essential feature of the model presented in Results and the Appendix is that the occupancy of Na^+ channels by TTX or STX is not only determined by this intrinsic affinity change but in addition by the extrinsic rate constants of toxin binding to the receptor. Thus, a sudden increase of the toxin affinity of the channel receptors does not produce an immediate but a delayed decline of the Na^+ current, owing to the slow binding of toxin molecules to the receptor. The 'on' and 'off' rate constants of TTX and STX binding to Na^+ channels in frog myelinated nerve were determined from Na^+ current measurements

after a fast change of the toxin concentration in the extracellular solution [18,19]. The published values of k_{on} and k_{off} have been used as first choice in the computation of normalized Na^+ currents (Fig. 6) and have been subsequently modified to yield the best agreement with the experimental results presented in Fig. 4.

Differences between TTX and STX

The fit and model parameters listed in Table I reveal remarkable differences between the monovalent cation TTX and the divalent cation STX: In general, the 'on' process is faster, the relaxation time constant, τ^*, is shorter and the magnitude of the affinity increase, Δk_{on}, is larger in the STX experiments. The rate constants k_{off} in Table I are published values as determined by rapid solution exchanges [18,19]. The k_{on} values from these experiments are $4 \cdot 10^6\ M^{-1} \cdot s^{-1}$ for TTX and $1.01 \cdot 10^7\ M^{-1} \cdot s^{-1}$ for STX and thus of the same order as the k_{on} data in Table I. The time constants, τ^*, of the affinity relaxation are one order of magnitude larger for TTX than for STX. Hence, the transition of the channel molecule to the 'normal state' is slow for the monovalent TTX and fast for the divalent STX. This suggests electrostatic interactions between the toxin cations and the channel receptor. Similarly, electrostatic effects of TTX and STX on Na^+ channels and differences between both toxins have been found in measurements of gating currents in crayfish axons [20].

Effects of holding potential on Na^+ channel receptors

In the crayfish axon [8] and in myelinated nerve (this paper, Figs. 1 and 2) use-dependent blockage of Na^+ channels by TTX or STX has been only observed at negative holding potentials. In heart muscle, on the other hand, the magnitude of the use-dependent TTX and STX effects does not seem to depend on the holding potential [4]. The underlying differences in the structure of Na^+ channels in axons and heart muscle are not yet known, but they are probably related to the high- and low-affinity toxin receptors in these tissues (see Introduction). The holding potential could not only affect the magnitude of the use-dependent TTX and STX effects in axon membranes but, in addition, also the toxin binding under holding conditions. A discrimination between stimulus-dependent and -independent effects of holding potential on TTX and STX binding to Na^+ channels requires a different type of experiment, the results of which will be presented in the following paper [21].

An effect of holding potential is not only seen for the TTX and STX binding sites in axon membranes but also for other external receptors on the Na^+ channel. Thus, the following inactivation modifying agents exert smaller effects at more positive holding potentials: Toxin ATX II from sea anemones [22], polypeptides from the snail *Conus* [23] and a coral [24], and scorpion toxins [25]. Such voltage-dependent affinity changes could be interpreted by membrane receptors located within the electric membrane field or by modifications in these receptors triggered by other field-sensitive channel structures.

Toxin binding and Na^+ channel gating

The affinity of channel receptors may not only be modified by long-lasting changes of the holding potential but also by short test pulses. As explained above, a single activation of the Na^+ channel could trigger an affinity increase of the TTX and STX receptor at negative holding potentials. Furthermore, TTX-blocked Na^+ channels in the squid giant axon exhibit a higher affinity to quaternary derivatives of lidocaine [26], TTX bound to Na^+ channels in heart muscle affects their gating properties [4], and STX increases the fraction of slowly inactivating Na^+ channels in frog myelinated nerve [27]. All these interactions between toxin binding and channel properties are readily understandable, since the main α-subunit of the Na^+ channel has been shown to contain not only the field sensor and the gate but also the TTX and STX binding site [28]. The consequences for the interpretation of experiments with repetitive test pulses in the presence of TTX or STX will be discussed in more detail in the following paper [21].

Appendix

A model for the use-dependent TTX and STX effects

The results illustrated in Figs. 1, 4 and 5 suggest that at negative holding potentials the equilibrium dissociation constant, K_d, of TTX or STX binding to Na^+ channels declines rapidly during a depolarizing test pulse and thereafter relaxes slowly to the initial value. A decline in $K_d = k_{off}/k_{on}$ can be caused by an increase in the forward rate constant, k_{on}, or by a decrease of the backward rate constant, k_{off}, in the toxin-receptor reaction (see Eqn. 3). For simplicity, it is assumed that k_{off} is time-independent and that the kinetics of k_{on} at a time t after the test pulse can be described by:

$$k_{on}(t) = \Delta k_{on} \cdot e^{-t/\tau^*} + k_{on} \tag{4}$$

with $\Delta k_{on} > 0$. This expression has to be inserted into the differential equation:

$$dy/dt = -(k_{off} + k_{on}(t) \cdot [T]) \cdot y + k_{off} \tag{5}$$

for the fraction y of unoccupied toxin receptors at the toxin concentration [T].

Eqns. 4 and 5 were solved numerically with the initial condition:

$$y(0) = k_{off}/(k_{off} + k_{on} \cdot [T]) \tag{6}$$

and with assumed values of the four model parameters k_{on}, Δk_{on}, k_{off} and τ^*. Since the variable y is proportional to the peak Na^+ current, the computed kinetics of $y(t)$ can be directly compared with the normalized peak currents I_i/I_0 in Fig. 4.

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