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

## II. Extrinsic influence of cations

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**Abstract.** Use-dependent declines of $Na^+$ currents in myelinated frog nerve fibres were measured during a train of depolarizing pulses in solutions containing tetrodotoxin (TTX) or saxitoxin (STX). The following effects of external monovalent ($Na^+$), divalent ($Ca^{2+}$, $Mg^{2+}$) and trivalent ($La^{3+}$) cations on use dependence were found: Increasing the $Ca^{2+}$ concentration from 2 to 8 m*M* shifts its voltage dependence by 20 mV whereas no significant use-dependent decline occurred at 0.2 m*M* $Ca^{2+}$. Doubling the external $Na^+$ concentration in 0.2 m*M* $Ca^{2+}$ solutions did not initiate phasic block. External $Mg^{2+}$ ions induced a smaller, and $La^{3+}$ ions a larger, use dependence. The time constants of the current decline were 4-fold greater in 1.08 m*M* $La^{3+}$. The static block of $Na^+$ currents by $La^{3+}$ could be directly demonstrated by the relief of block during a train of pulses. The results are qualitatively explained by a toxin binding site at the $Na^+$ channel whose affinity for TTX or STX depends on *i*) the gating conformation of the channel, probably the inactivation and *ii*) the occupancy of a blocking site by di- or trivalent external cations.

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

## Introduction

The recently discovered use-dependent blockage of $Na^+$ channels by guanidinium toxins at the crayfish axon (Salgado et al. 1986) and in myelinated nerve (Lönnendonker 1989 a) cannot be explained solely by the modulated receptor hypothesis (reviewed in Hille 1984). According to this hypothesis the receptor of the $Na^+$ channel for local anaesthetics or for other drugs has at least three major states (resting, activated, inactivated) differing in their drug-binding affinities and rate constants. In contrast, the use-dependent action of tetrodotoxin (TTX) and saxitoxin (STX) on $Na^+$ channels in axon membranes also depends on external cations (Salgado et al. 1986). Thus, the use-dependent decline of $Na^+$ currents during a train of depolarizing test pulses is the result of state-dependent 'access' of the toxin to a binding site (Lönnendonker 1991 b) whose toxin affinity is modulated by external cations. These complicated interactions between the gating states of the $Na^+$ channel and cations of the external solution are the subject of this paper. It is shown that a description of the toxin action must take into account the holding potential, the amount of toxin used and the concentration of mono- and polyvalent cations. Some of these results have been published in abstract form (Lönnendonker 1991 a).

## Materials and methods

Most of the methods are described in the companion paper (Lönnendonker 1991 b). All experiments were performed at 15 °C.

### *Solutions*

As stated in Lönnendonker (1991 b) the external solution (Ringer) was composed of 110 m*M* NaCl, 2 m*M* $CaCl_2$, 10 m*M* tetraethylammonium chloride (TEA-Cl), 4 m*M* MOPS and 8 n*M* tetrodotoxin (TTX) or 8 n*M* saxitoxin (STX). In some experiments the $CaCl_2$ concentration was reduced (0.2 m*M*) or raised (8 m*M*). In other experiments $CaCl_2$ was substituted by 3 or 8 m*M* $MgCl_2$. The external solution in the $La^{3+}$ experiments contained 110 m*M* NaSCN, 1.08 m*M* $LaCl_3$, 10 m*M* TEA-Cl and 4 m*M* MOPS (NaSCN-Ringer). In another series of experiments the $Na^+$ concentration was raised to 220 m*M* and only 0.2 m*M* $CaCl_2$ was added.

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*Analysis*

During a train of depolarizing pulses the peak $Na^+$ currents decline from an initial value $I_0$ to a steady-state value $I_\infty$. The ratios $I_\infty/I_0$ as function of the holding potential $V_H$ were analysed by two different methods. The linear part of the dependencies could be described by a regression line. In addition, the assumed sigmoid curves are fitted with Eq. (2) of Lönnendonker (1991 b) giving the inflection point and the steepness factor of the curve. The voltage shifts of the inflection points in Table 1 were comparable to the displacement of the curves estimated by eye.

In four fibres the voltage dependencies of the parameters of $Na^+$ activation $m_\infty, \tau_m$ and inactivation $h_\infty, \tau_h$ were determined for Ringer and NaSCN-Ringer (see Fig. 4) with methods described previously (Neumcke and Stämpfli 1982). In short, the time constants $\tau_m$, $\tau_h$ of $Na^+$ activation and of fast $Na^+$ inactivation were found by fitting the expression:

$$I(t) = I'(1-\exp(-t/\tau_m))^3 \exp(-t/\tau_h) \quad (1)$$

to the $Na^+$ current $I(t)$. The steady-state values $m_\infty$ of $Na^+$ activation were derived from the fitted parameters $I'$ which were converted into $Na^+$ permeabilities by means of the constant field equation (Frankenhaeuser 1960) and divided by the extrapolated maximum $Na^+$ permeability to yield the quantity $m_\infty^3$. The steady-state inactivation was measured with 50 ms prepulses between $V_p = -50$ and 50 mV and test pulses to 60 mV.

**Table 1.** Voltage shifts of use dependence and gating parameters. Shifts of use dependence are derived from fits of Eq. [2] of Lönnendonker (1991 b) to $I_\infty/I_0$ values vs. $V_H$ and calculated as differences of inflection points with respect to a $Ca^{2+}$ concentration of 2.0 m*M*. Other shifts are taken from + Hille et al. (1975), # Hille (1968) without toxin or are from own exeriments with toxin (see text). The values * are estimated from upper and lower limits

| Cation type | Conc. | Toxin | 'Shifts' in mV | | |
|---|---|---|---|---|---|
| | [m*M*] | [8 n*M*] | use dep. | $h_\infty$ | $m_\infty$ |
| Ca | 2.0 | STX/TTX | 0 | 0 | 0 |
| | 0.2 | STX | $<-25$* | | $-12^+$ |
| | 8.0 | STX | 21.7 | $>10^\#$ | $12^\#$ |
| Mg | 3.0 | STX | 5.9 | | $0^+$ |
| | 8.0 | STX | 14.5 | | $6^+$ |
| La + | 1.08 | STX | 36.7 | 15 | 7 |
| NaSCN | 1.08 | TTX | $>20$* | | |

## Results

*$Ca^{2+}$ concentration dependence*

It was already noticed by Salgado et al. (1986) that the use-dependent effects are not only affected by the holding potential but also depend on the $Ca^{2+}$ concentration of the external solution. Figure 1 shows $I_\infty/I_0$ values vs. the holding potentials for a normal $Ca^{2+}$ concentration of 2 m*M* (A) and for a reduced or raised concentration (B) in external solutions with 8 n*M* STX. The steepness factors from the data in Ringer with 2 m*M* $Ca^{2+}$ (compare Lönnendonker 1991 b) were $-17.15$ and $-12.33$ mV for TTX and $-7.21$ and $-9.23$ mV for STX, with 4 and 8 n*M* toxin respectively. The increase in $Ca^{2+}$ did not change the steepness of the $I_\infty/I_0$ vs. $V_H$ relation, rather it shifted it to more positive potentials as expected for surface potential effects. The absence of a significant use-dependent current decline at a lower $Ca^{2+}$ concentration of 0.2 m*M* (symbols o in Fig. 1 B) was striking. It cannot, however, be excluded that such declines could be observed at more hyperpolarizing holding potentials, not reached in these experiments. Table 1 compares fit values (Eq. (2) of

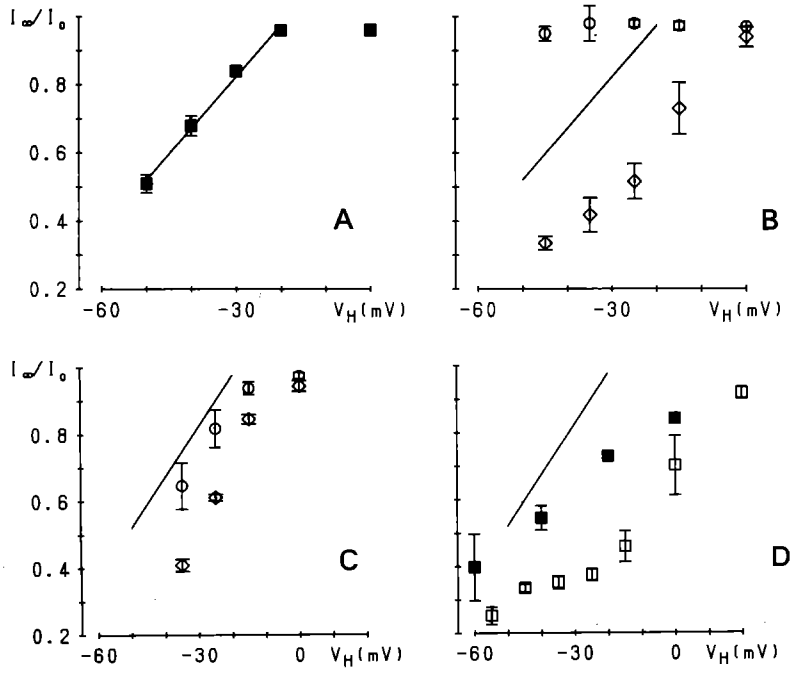


**Fig. 1 A–D.** Ratios of stationary ($I_\infty$) and initial ($I_0$) peak $Na^+$ currents during depolarizations to $V = 60$ mV vs. the holding potential $V_H$. Symbols and bars denote means ±SEM. The solutions contain different amounts of cations and 8 n*M* STX (*D* ■ with 8 n*M* TTX). The regression line from **A** is repeated in **B, C** and **D**. The external solutions at 15 °C contained 2 m*M* $Ca^{2+}$ (■, **A**), 0.2 m*M* $Ca^{2+}$ (o, **B**) or 8 m*M* $Ca^{2+}$ (◇, **B**), **C** two different $Mg^{2+}$ concentrations of 3 m*M* (o) and 8 m*M* (◇). **D** NaSCN-Ringer with a $La^{3+}$ concentration of 1.08 m*M* instead of $Ca^{2+}$ and with 8 n*M* STX (□) or 8 n*M* TTX (■). Note the absence of a significant use-dependent effect for 0.2 m*M* $Ca^{2+}$ even at a holding potential of $V_H = -45$ mV. Pulse frequency 1 Hz

Lönnendonker 1991 b) of the shifts of $I_\infty/I_0$ curves with shifts of steady-state activation and inactivation values respectively. It is evident that these 'use-dependent' shifts were significantly larger than shifts of activation and inactivation parameters. It is, therefore, probable that such shifts were only partially induced by surface potential changes. The steepness of the $I_\infty/I_0$ curves in a solution with 8 m$M$ $Ca^{2+}$ was $-8.05$ mV (regression analysis $0.0139 \pm 0.0041$ $I_\infty/I_0$ per mV), and thus not changed relative to the $-9.23$ mV in 2 m$M$ $Ca^{2+}$.

The time constants $\tau$ of the current decline obtained in a solution with the higher $Ca^{2+}$ concentration of 8 m$M$ ($14.4 \pm 0.53$ s, mean $\pm$SEM) were larger than the values in normal $Ca^{2+}$ and at the same amount of STX ($9.2 \pm 1.8$ s). The divalent cation $Ca^{2+}$ had, therefore, profound effects on the kinetics of use dependence with STX.

### *Replacement of $Ca^{2+}$ by $Mg^{2+}$*

To test whether $Ca^{2+}$ had a specific effect on the use dependence, I replaced $Ca^{2+}$ by $Mg^{2+}$. $Mg^{2+}$ is the divalent cation with the smallest static blocking effects on $Na^+$ currents known (Grissmer 1984). In Figs. 1 C, 2 A and Table 1, the effects of two different $Mg^{2+}$ concentrations are summarized. The $I_\infty/I_0$ values (Fig. 1 C) were only shifted in solutions containing 8 n$M$ STX. These shifts compiled in Table 1 were again higher than the shifts of activation parameters. The steepnesses of the $V_H$ dependencies were $-8.93$ and $-8.25$ mV (regression analysis $0.015 \pm 0.019$ and $0.022 \pm 0.012$ $I_\infty/I_0$ per mV) for solutions with 3 and 8 m$M$ $Mg^{2+}$ respectively. Both parameters showed no significant 'slope' changes compared to the values in $Ca^{2+}$.

It is noteworthy that the time constants $\tau$ of the decline in 3.0 m$M$ $Mg^{2+}$ ($9.3 \pm 1.0$ s) and especially in 8.0 m$M$ $Mg^{2+}$ ($13.4 \pm 1.2$ s) were higher than in solutions with 2.0 m$M$ $Ca^{2+}$ (9.2 s) and again were nearly independent of the holding potential (Fig. 2 A). Thus, the time constants also seemed to be dependent on the type of divalent cation used.

### *The trivalent cation $La^{3+}$*

The trivalent cation $La^{3+}$ at a concentration of 1.08 m$M$ shifts the gating parameters of $Na^+$ channels by $+30$ mV along the voltage axis (Vogel 1974; Brismar 1980; Grissmer 1984). In contrast, the monovalent anion $SCN^-$ (110 m$M$) induces an opposite voltage shift of $-20$ mV (Grissmer 1984; Neumcke and Stämpfli 1986). To exclude such dramatic shifts which would also affect the voltage dependence of the use dependence, I used an external solution of 110 m$M$ NaSCN with 1.08 m$M$ $La^{3+}$ (see Materials and methods). With a linear superposition of the shifts mentioned it is expected that the resulting voltage shift would be only $+10$ mV.

Figure 3 B shows the drastic decline of $Na^+$ currents in NaSCN-Ringer $+La^{3+}$ and 8 n$M$ STX at a holding potential of $V_H = -55$ mV. Notice the slow activation and inactivation of the currents (compare Fig. 4) and the small current amplitudes. The latter is known to be a sign of a static block of $Na^+$ currents by $La^{3+}$ (Vogel 1974; Grissmer 1984). The same external solution without STX did not elicit such a decline (Fig. 6 A and control experiments with different holding potentials).

To determine the properties of $Na^+$ channels in this external solution, current-voltage relations and gating parameters were measured. To exclude series resistance differences between the control and this external solution, the control was measured in Ringer with 16 n$M$ STX and the test solution contained 8 n$M$ STX. This gives nearly identical current amplitudes at corresponding voltages. Another unavoidable problem was a use-dependent decline of the currents while performing the measurements. Therefore, the holding potential was set to $V_H = 0$ mV to reduce use-dependent influences (compare Fig. 3 A). In Fig. 4 all gating parameters determined for NaSCN-Ringer $+La^{3+}$ on one fibre are compared with values obtained in Ringer. The steady-state inactivation values in NaSCN-Ringer and $La^{3+}$ (Fig. 4 C) were shifted by $+20$ mV at positive prepotentials and depressed at hyperpolarizing prepotentials. A smaller voltage shift of $+2$ mV was observed for the steady-state activation variables (Fig. 4 A). The activation time constant, $\tau_m$ was shifted by a larger amount than the inactivation time constant $\tau_h$. Similar results were reported by Gilly and Armstrong (1982) for the effects of $Zn^{2+}$ on activation (shift $+20$ to $+30$ mV) and inactivation kinetics (shift 0 to $+6$ mV). In my experiments the following mean voltage shifts were found: (1) the reversal potentials of the peak $Na^+$ current-voltage curves were shifted by $-3.25 \pm 1.5$ mV, the steady-state inactivation curve by $15.1 \pm 0.73$ mV and the descending branch of the current-

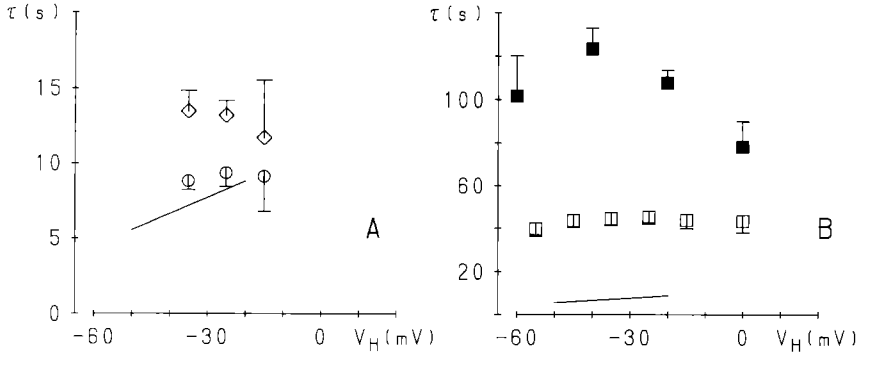


**Fig. 2 A, B.** Time constants $\tau$ (means $\pm$SEM) of the decline of peak $Na^+$ currents for two different $Mg^{2+}$ concentrations of 3 m$M$ (○) and 8 m$M$ (◇) in **A** and one $La^{3+}$ concentration (1.08 m$M$) in **B**. $Na^+$ currents are partially blocked by 8 n$M$ STX (○, ◇, □) or 8 n$M$ TTX (■). The regression line are from experiments in solutions with 2 m$M$ $Ca^{2+}$ and 8 n$M$ STX to enable comparison (compare Lönnendonker 1991 b)

voltage curve by 7.29±0.91 mV (mean ±SEM, 4 fibres and 6 to 8 values). In particular, inactivation was more influenced than activation (compare Table 1).

The use dependence obtained in NaSCN-Ringer $+La^{3+}$ was very strong. As can be seen in Fig. 1D the $I_\infty/I_0$ values were shifted by +37 mV in 8 n*M* STX (symbols □) and by more than +20 mV in solutions with 8 n*M* TTX (symbols ■, see Table 1). Moreover, the fibres were more stable in NaSCN-Ringer and the holding-potential dependence could be resolved at more hyperpolarized potentials. The steepness of the $V_H$-dependence of $I_\infty/I_0$ was around −10 mV (regression analysis 0.0076± 0.002 $I_\infty/I_0$ per mV) for TTX and −11.47 mV (regression for values at $V_H = 20$ mV to −15 mV: 0.0129±0.019 $I_\infty/I_0$ per mV) for STX containing external solutions. As shown in Fig. 2B the time constants of decline in 8 n*M* TTX (mean $\tau = 102.5 \pm 6.9$ s, symbols ■) were larger by a factor of 2.4 than in 8 n*M* STX ($\tau = 43.8 \pm 1.4$ s, symbols □; compare Lönnendonker 1991 b). Moreover, both time constants were 4-(6)-fold greater in NaSCN-Ringer $+La^{3+}$ and 8 n*M* TTX (STX) compared to Ringer with the same toxin concentration.

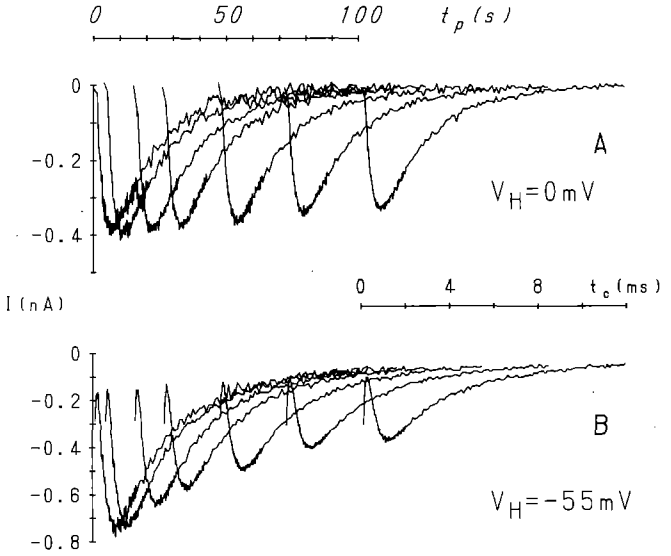


**Fig. 3A,B.** $Na^+$ currents during a train of depolarizing test pulses to $V = 60$ mV. The external solution contained 1.08 m*M* $La^{3+}$ (instead of 2.0 m*M* $Ca^{2+}$), 110 m*M* NaSCN and 8 n*M* STX. Recordings at two different holding potentials $V_H$ as indicated. Shown are the mean currents after the pulse time $t_p$ displaced against each other. The axis $t_c$ gives the time for each individual recording

## *Frequency dependence*

It is noteworthy that the use dependence of STX in NaSCN-Ringer $+La^{3+}$ was also dependent on the gap time between subsequent pulses, whereas in 8 n*M* TTX it was independent in the range of frequencies tested (Fig. 5). This result was surprising as the increase of the time constant $\tau$ against Ringer (Lönnendonker 1991 b) was not accompanied by a change in the kinetic differences between STX and TTX. Thus this difference between both toxins must be an inherent 'kinetic' property of STX and TTX.

## *Relief of block*

The currents in NaSCN-Ringer $+La^{3+}$ were significantly smaller than in Ringer because $La^{3+}$ blocks $Na^+$ channels in myelinated nerve (Vogel 1974; Grissmer 1984). The relief from this block is illustrated in Fig. 6 which depicts means of several normalized peak current trains. The control without toxin in the external solution (Fig. 6A) showed a long lasting but small increase of the peak current. This increase can also be seen in Fig. 6B transiently for the mean values with TTX (symbol ○), but not for STX containing solutions (symbol △). The increase in peak current in solutions without and with 8 n*M* TTX reached, in both cases, only 2.2% of the peak at $t_0$. Under different conditions such a transient increase could be obtained even in external solutions with STX (compare Fig. 5A).

## *$Na^+$ does not 'replace' $Ca^{2+}$*

Evidently, the previous results suggest that various cations 'compete' with toxins for a binding site: the more

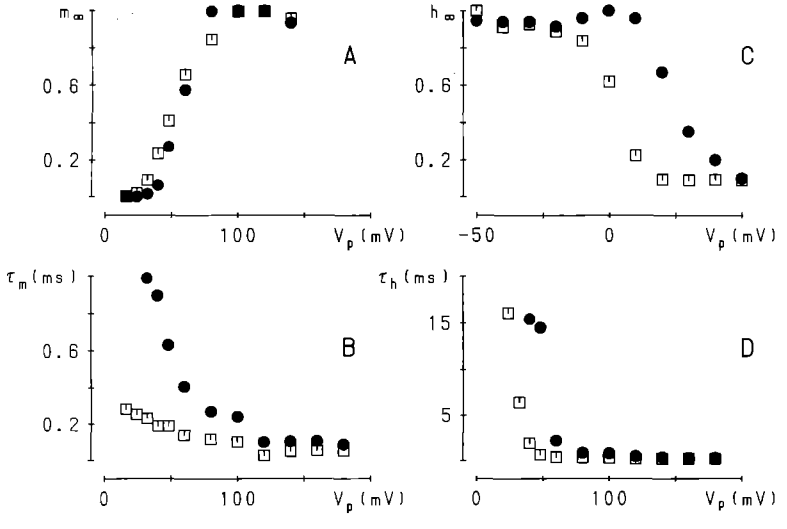


**Fig. 4A–D.** Parameters of $Na^+$ activation ($m_\infty$, **A** and $\tau_m$, **B**) and inactivation ($h_\infty$, **C** and $\tau_h$, **D**) as function of depolarization $V_p$. Measurements in Ringer (□) and NaSCN-Ringer with 1.08 m*M* $La^{3+}$ (●). The measurements were performed on one fibre at the holding potential $V_H = 0$ mV. The external solution contained 16 n*M* STX (□) or 8 n*M* STX (●) to get nearly identical peak sodium currents. Note the different scaling of the abszissa in **C**. All measurements from one fibre

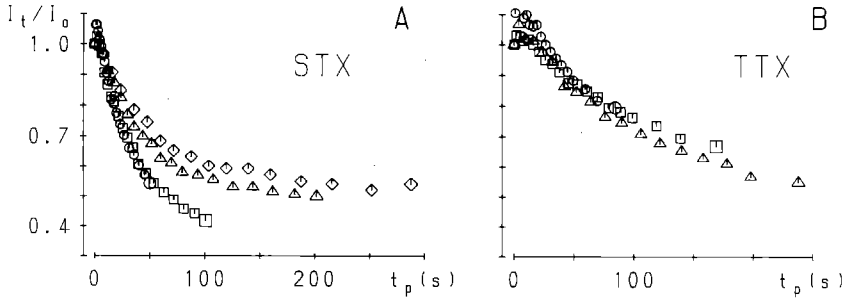


**Fig. 5 A, B.** Normalized peak $Na^+$ currents $I_t/I_0$ at different pulse frequencies in an external solution of NaSCN-Ringer with 1.08 m*M* $La^{3+}$ and 8 n*M* STX (**A**) or 8 n*M* TTX (**B**). The pulse frequencies were: 0.25 Hz (◇), 0.5 Hz (△), 1 Hz (□) and 2 Hz (○). Note that currents in STX solutions are more dependent on the pulse frequency and the transient increase in the currents beginning at the second pulse. Holding potential $V_H = -40$ mV

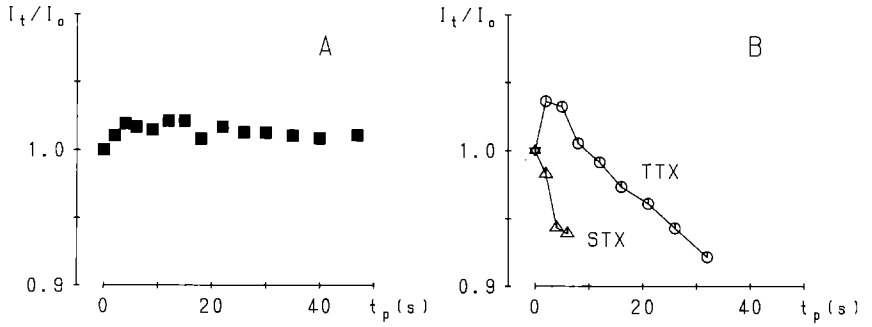


**Fig. 6 A, B.** Means of normalized peak $Na^+$ currents, $I_t/I_0$ vs. pulse time, $t_p$ in experiments with an external solution of NaSCN-Ringer with 1.08 m*M* $La^{3+}$. The frequency of pulses was 1 Hz. **A** External solution without toxin (■, $V_H = -40$ mV); **B** solutions with 8 n*M* TTX (○, $V_H = -40$ mV) or 8 n*M* STX (△, $V_H = -45$ mV). Means from three measurements on one fibre (**A**) and five experiments (three fibres) in **B**

strongly bound trivalent $La^{3+}$ or the divalent $Ca^{2+}$ or $Mg^{2+}$ ions. It was also shown previously that $Na^+$ ions interfere with STX-block (Lönnendonker et al. 1990), and Salgado et al. (1986) have proposed that $Na^+$ can be trapped in the pore mouth. Thus I tested whether $Na^+$ can induce use-dependent effects with an external solution containing a high concentration of NaCl (220 m*M*) and a low concentration of $Ca^{2+}$ (0.2 m*M* which elicits no use dependence, see Fig. 1 B symbols ○). However, this excess of monovalent $Na^+$ ions could not induce any use dependence of STX by itself in a range of holding potentials $V_H$ between −30 and 0 mV (not shown).

## Discussion

### *Surface potential*

An alteration of the surface potential is considered to be the main source of the voltage shifts of gating variables found by changing the cation or anion concentration in the external solution. Frankenhaeuser and Hodgkin (1957) first proposed that binding to or screening of surface charges by $Ca^{2+}$ ions changes this potential. The shifts of gating variables compiled in Table 1 are only an estimate of the surface potential changes because we cannot measure these potentials directly. However, the effective part of the shift 'seen' by *m* or *h* gates can be calculated. For example, 'competition' between guanidinium toxins and cations or anions reveals that the toxin binding site should feel only 46% to 20% of the voltage shifts (Grissmer 1984; Neumcke and Stämpfli 1986). All 'shifts' for use-dependent declines (Table 1) are greater than the shifts of the gating variables. Moreover, the charge difference between the divalent $Ca^{2+}$ and the trivalent $La^{3+}$ cannot explain a shift of 37 mV at comparable cation concentrations. Hence, it would be difficult to explain these effects with changes in surface potential alone.

### *Cations affect toxin binding and other parameters*

That cations directly affect toxin binding could be demonstrated several times in different preparations (reviewed in Strichartz et al. 1986). Recently it was shown for $Na^+$ and STX or TTX (Ravindran and Moczydlowski 1989) and for other monovalent cations and STX (Lönnendonker et al. 1990). Krueger et al. (1986) reported competition of $Na^+$ and $Ca^{2+}$ and STX for the same binding site, and Worley et al. (1986) found a reduction of trimethyloxonium-modification (TMO) of the toxin site by an increased $Ca^{2+}$ concentration. Krueger et al. (1986); Green et al. (1987 a, b); and Pusch (1990) also discuss screening and several binding sites for the $Ca^{2+}$ action on toxin binding. Thus the tonic and the phasic effects on toxin binding induced by cations in my experiments could both be produced at least partially by a competition between TTX or STX and external cations. In addition, cations interact directly with a negatively charged group of the gating apparatus (Gilly and Armstrong 1982; Conti et al. 1976; Hille 1984). The differences in shifts of the kinetics and steady-state values of activation and inactivation found here for NaSCN + $La^{3+}$ solution (Fig. 4) could be an example of such a binding. Also, the 'shifts' of the use dependence (Table 1) must be produced by such 'binding' effects of cations.

### *Reduction of $Na^+$ permeability*

Another action of externally applied divalent and trivalent cations was a reduction of $Na^+$ peak current ampli-

tude (compare Fig. 3A of this paper). Of the divalent cations, only $Ca^{2+}$ might be slightly permeant in $Na^+$ channels of frog nerve ($P_{Ca}/P_{Na} < 0.11$, Vogel 1974). All polyvalent cations exert a blocking action on $Na^+$ channels. Thus, Grissmer (1984) reported for toxin-free solutions reductions of the $Na^+$ permeability to 90% by 10 m*M* $Mg^{2+}$, to 70% by 1.08 m*M* $La^{3+}$ and to 80% in external solutions containing the anion $SCN^-$ in place of $Cl^-$. Krueger et al. (1986); Yamamoto et al. (1984); and Sheets et al. (1987) found a $Ca^{2+}$ block of $Na^+$ channels in different preparations which is enhanced by hyperpolarization. A voltage-dependent channel block by $La^{3+}$ is shown in Fig. 4C where the $h_\infty$ curve at hyperpolarizing prepotentials is depressed by a stronger binding of $La^{3+}$ to the $Na^+$ channel. Another explanation for such a depression could be a delayed activation of the current (Chiu 1977). Recently, $Zn^{2+}$ induced subconductance events were obtained in BTX-modified cardiac $Na^+$ channels (Ravindran et al. 1991).

Figure 6A illustrates the relief of block by $La^{3+}$ ions. A similar relief of block by external $Ba^{2+}$ in $K^+$ channels was reported by Armstrong et al. (1982) for the squid axon. In the presence of TTX or STX (Figs. 5, 6B) the toxin binding sites at $Na^+$ channels were occupied only transiently by the toxins resulting in a short-lived increase of peak $Na^+$ currents. In TTX containing solutions this decrease is slower than for STX, in agreement with the different toxin kinetics (Ulbricht and Wagner 1975; Wagner and Ulbricht 1975).

### *A qualitative model of use dependence*

Any model of use-dependent block of $Na^+$ channels by TTX or STX must explain its dependence on conditioning holding potentials (Fig. 1). There are three possibilities for how the membrane voltage can influence use dependence: a) the toxin binding site of the channel is directly influenced by the voltage, b) the affinity for the toxin depends on the gating conformation of the channel or c) voltage-dependent binding of external cations ($Ca^{2+}$, $La^{3+}$) influences the phasic toxin block indirectly. Since the channel block by $Ca^{2+}$ ions is known to be voltage dependent (Woodhull 1973) and the static toxin block is probably voltage independent (Lönnendonker 1989b), the indirect mechanism (c) is more likely (compare Figs. 1, 2 and 6). I, therefore, propose that the divalent or trivalent cations bind to a 'deep' site ('cation site') in the channel thereby reducing TTX or STX binding to an external receptor. The binding by these cations can only partially block the channels because it is assumed to be a fast flickery block with rapid binding and unbinding kinetics. This picture is reminescent of the 'divalent gating particle theory' which was discussed by Frankenhaeuser and Hodgkin (1957). However, with guanidinium toxins the binding and relief of flickery block by the cations ($Ca^{2+}$, $La^{3+}$) does not gate the channel, but additionally 'closes and opens' a toxin binding site. This site must be 'opened' for the toxins by depolarizations to explain the use-dependent effects and the involvement of $Na^+$ inactivation in use dependence (Lönnendonker 1991 b).

This model can account qualitatively for the use-dependent block of $Na^+$ channels by TTX or STX, but some further assumptions are required to explain the following quantitative results: *i*) At higher divalent cation concentrations or with $La^{3+}$ ions in the external solution the time constants $\tau$ of the development of use dependence (Fig. 2) were always higher than the values $\tau_{on}$ of toxin binding in Ringer (Ulbricht and Wagner 1975; Wagner and Ulbricht 1975). This suggests that the number of free binding sites increases slowly during a repetitive pulse protocol (Fig. 6A). *ii*) Replacement of external divalent ($Ca^{2+}$, $Mg^{2+}$) by trivalent ($La^{3+}$) cations does not produce a large change of the steepness of the voltage dependence of use dependence (Fig. 1D), which is difficult to reconcile with a fixed cation binding site within the $Na^+$ channel (but compare Ravindran et al. 1991).

In conclusion, all results point to an involvement of $Ca^{2+}$ binding to a 'deep' site in the $Na^+$ channel in use-dependent channel block by TTX or STX, but the magnitude of the effects cannot be fully explained.

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