J. Membrane Biol. 106, 29–40 (1988)

The Journal of
Membrane Biology

Mechanism of Inactivation of Single Sodium Channels after Modification by Chloramine-T, Sea Anemone Toxin and Scorpion Toxin

Karoly Nagy*
I. Physiologisches Institut der Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

Summary. Single sodium-channel currents were measured in neuroblastoma cells after inhibition of inactivation by chloramine-T (CHL-T), sea anemone toxin II (ATX-II) and scorpion toxin (SCT). The decaying phase of the averaged single-channel currents recorded with 90-msec pulses in cell-attached patches was clearly slower than that of the unmodified channels, suggesting inhibition of macroscopic inactivation. Each substance caused repetitive openings and a moderate increase in the channel open time. At $V_m = RP + 20$ mV and $T = 12$°C, the mean channel open times were 1.4, 1.6 and 1.8 msec for CHL-T, ATX-II and SCT, respectively, as opposed to 1.07 msec for native channels. Open-time histograms could be best fitted by the sum of two exponentials. The time constants of the fits were similar for histograms constructed from single openings and from openings during bursts. This suggests that the population of channels is homogeneous and that in bursts the same open conformations of channels occur as in single openings. Mean burst durations for bursts consisting of more than one opening at $V_m = RP + 20$ mV were 4.9, 5.8 and 6.1 msec for CHL-T, ATX-II and SCT, respectively. Burst open-time histograms constructed from two or three openings were fitted by the gamma function. The different time constants of the fits obtained for ATX-II and SCT suggested multiple open conformations of channels for openings of bursts. However, significantly different open-time histograms constructed from the first, second and third openings of bursts could not be obtained systematically. A positive correlation was found for the dwell time of the first and the second, as well as for the second and the third opening of bursts with each substance, but a negative one for the dwell time of an opening and the neighboring closing of bursts with ATX-II. The results suggest a model with multiple open and inactivated states. In this model the inactivated states are weakly absorbing.

Key Words single sodium channels · neuroblastoma cell · chloramine-T · sea anemone toxin · scorpion toxin · inhibition of inactivation · burst openings

Introduction

Inactivation of the macroscopic sodium current can be inhibited by various substances, e.g., scorpion toxin (Koppenhofer & Schmidt, 1968; Meves, Rubly & Watt, 1982; Meves, Simard & Watt, 1984; Wang & Strichartz, 1985; Gonoi & Hille, 1987), sea anemone toxin (Bergman et al., 1976; Neumcke, Schwarz & Stampfli, 1985; Ulbricht & Schmidtmayer, 1981; Meves et al., 1984), chloramine-T (Wang, 1984; Wang et al., 1985; Rack, Rubly & Waschow, 1986). Although the effect of these substances on macroscopic inactivation is similar in different preparations, the effect on microscopic inactivation could be different. On the single-channel level, sea anemone toxin has been shown to prolong the channel open time and cause repetitive channel opening ("bursting") in cardiac muscle (Schreibmayer, Kazerani & Tritthart, 1987). In neuroblastoma cells it also causes bursts, but the channel open time is only moderately increased (Nagy, 1987*d*). Chloramine-T elicits bursting too, but its effect on the channel open time is small (Nagy, 1987*d*) or variable (McCarthy & Yeh, 1987).

This paper presents a detailed analysis of the effects of chloramine-T (CHL-T), sea anemone toxin (ATX-II) and scorpion toxin (SCT) on single sodium-channel currents of neuroblastoma cells. The results suggest the existence of multiple open and inactivated states. The inactivated states are moderately absorbing making the reopening of the channels possible. Parts of the results have been reported in a preliminary form (Nagy, 1987*b*,*c*).

Materials and Methods

Single sodium-channel currents were measured in mouse neuroblastoma cells, N1E 115, by the patch-clamp method in the cell-attached configuration (Hamill et al., 1981). Cells were grown under standard conditions as described by Moolenaar and Spector (1978). Details of the experimental methods have been published (Nagy, Kiss & Hof, 1983; Nagy, 1987*a*) and will be summarized briefly.

Lipid-coated pipettes formed seals with a resistance of 30 to

* *Present address:* Institut für Biologie II, RWTH Aachen, Kopernikusstr. 16, D-5100 Aachen, FRG.

50 GΩ. The cell membrane was hyperpolarized by 50 to 80 mV to remove resting inactivation. From the constant holding potential, 40- to 90-msec depolarizing voltage pulses of different heights were applied at intervals of 1.0–1.5 sec.

During the experiments, cells were kept in either HEPES buffered medium or in a bath solution that contained (in mM): NaCl 160, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.8, HEPES 20, glucose 20. pH was adjusted to 7.3 and temperature (between 12 and 16°C) was kept constant during each experiment. Pipettes were filled with bath solution or a solution containing 160 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 5 mM HEPES.

In 13 experiments, cells were incubated in bath solution containing 0.5–1.0 mM CHL-T. After 5 to 8 min, the CHL-T solution was washed out and measurements were carried out in normal bath solution.

In 11 experiments, 3–7 μM ATX-II was added to the pipette solution. ATX-II from *Anemonia sulcata* was purchased from Ferring GmbH (Kiel, FRG).

In 10 experiments, 110–330 nM SCT was added to the pipette solution. The toxin was purified from the venom of the scorpion *Leiurus quinquestriatus* by Dr. M. Rack (Physiol. Chem. Homburg, FRG).

Due to the uncertainty of the cell's resting potential, parameters of different patches were not pooled or compared directly. Instead, long experiments with single patches were mainly used for the present analyses. If parameters of different patches (measured at the same temperature) are compared, the fast time constants of the first latency density functions, the times to peak of the averaged single-channel current records and the single-channel current amplitudes were used to recalculate the pulse potential. The latter method can be used because the single-channel conductances of the modified and unmodified channels are very similar (*see* Results). The three methods resulted in similar values for the pulse potential.

Small shifts of the activation parameters have been reported with the three substances in different preparations: with CHL-T, between 5 and 8 mV (Meves & Rubly, 1986; Rack et al., 1986; Drews, 1987), with ATX-II between 0 and −4 mV (Ulbricht & Schmidtmayer, 1981; Neumcke et al., 1985) and with SCT between 0 and −10 mV (Wang & Strichartz, 1985; Gonoi & Hille, 1987). A few millivolts change in the membrane potential can cause a few percent change in the channel-open or burst time parameters, which are compared in this paper.

Details of the measuring system have been described previously (Nagy et al., 1983; Hof, 1986; Nagy, 1987*a*) and are summarized briefly as follows. A DEC LSI 11/23 microcomputer generated the voltage pulses, sampled the data with 10 kHz and was used for off-line analyses. Analog signals were filtered at 2 kHz (−3 dB) by a four-pole low-pass Bessel filter. Leakage and capacitive currents were compensated by an analog circuit.

Data Analyses

Calculations and calibrations were made to determine the kinetic parameters of the recording system as described earlier (Nagy, 1987*a*). The dead time of the recording system was 0.09 msec and the signal-to-noise ratio was >8.2.

The half-amplitude threshold detection was used for calculations of the open, closed and burst open times of the channel openings with the accuracy of the sampling interval (0.1 msec) on records without overlapping openings. Open- and closed-time histograms were constructed with a bin width of 0.1 msec and burst-time histograms with 0.2 msec from channel openings on at least 200 current records measured with a certain pulse potential.

All histograms were fitted by a Gaussian least-squares fit routine. Open- and closed-time histograms were fitted by the sum of two exponential functions

$$f(t) = (w_1/\tau_1)\exp(-t/\tau_1) + (w_2/\tau_2)\exp(-t/\tau_2). \quad (1)$$

Here w_i is the weighting factor of the ith component, $w_1 + w_2 = 1$ and τ_i is the time constant. Chi-square values were calculated for each fit to estimate the number of exponential components of the histograms (Colquhoun & Sigworth, 1983).

Burst open times were defined as consecutive openings that are separated by closed intervals shorter than a specified time t_c. t_c was determined from the closed-time histograms using the criterion that the portion of long-closed intervals erroneously classified as gaps between bursts is equal to the portion of the misclassified short-closed intervals. This condition, which was also used by Colquhoun and Sakmann (1985), is fulfilled if

$$\exp(-t_c/\tau_1) = 1 - \exp(-t_c/\tau_2) \quad (2)$$

where τ_1 and τ_2 are the fast and the slow time constants of the closed-time histogram, respectively.

Burst open-time histograms were constructed separately from bursts consisting of two and three openings. These histograms were fitted by a gamma function (Colquhoun & Sakmann, 1985)

$$\Gamma_k(t) = (t/\tau)^{k-1}\exp(t/\tau)(\tau(k-1)!)^{-1}. \quad (3)$$

Here, τ is a mean time constant, k is the number of variables (in the present case 2 or 3).

The distribution of the number of closing per burst was fitted by a geometric function (Colquhoun & Sigworth, 1983; Colquhoun & Sakmann, 1985)

$$P(n) = (a_1/\mu_1)(1 - \mu_1^{-1})^{n-1} + (a_2/\mu_2)(1 - \mu_2^{-1})^{n-1}. \quad (4)$$

In this equation n is the number of closings per burst, a_i is the weight of the ith component and μ_i is the mean value of the ith component.

Results

General Observations

Chloramine-T irreversibly inhibits the inactivation of sodium channels in neuroblastoma cells (Nagy, 1987*b*; Nagy, 1987*c*), similar to the effect reported in myelinated nerve fibers (Wang, 1984) and squid axons (Wang et al., 1985). The effects of sea anemone toxin and scorpion toxin is reversible, as they are on other preparations (Strichartz, Rando & Wang, 1987).

Figure 1 shows single-channel current records from CHL-T, ATX-II and SCT modified sodium channels. The records demonstrate that the modified channels open not only shortly after the onset of the depolarization, but also later during the 90-

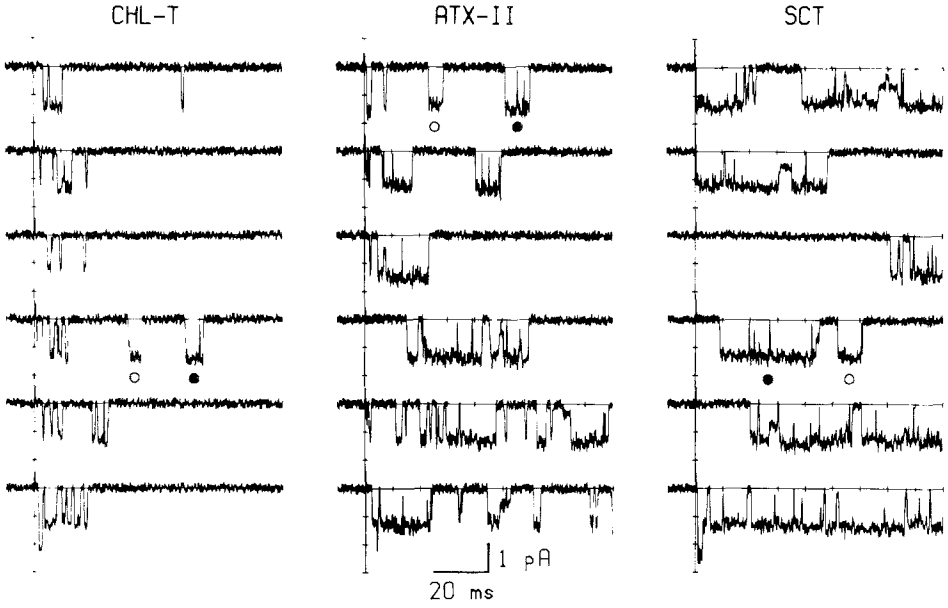


Fig. 1. Selected single-channel currents through sodium channels modified by chloramine-T (*CHL-T*), sea anemone toxin (*ATX-II*) and scorpion toxin (*SCT*) in cell-attached patches of neuroblastoma cells, N1E 115. $V_H = RP - 60$ mV for CHL-T and ATX, and $V_H = RP - 50$ mV for SCT. Patches were depolarized to $V_m = RP + 10$ mV for 90 msec, $T = 14$°C for each plot. Open and filled circles indicate single openings and openings in a burst, respectively

msec pulse to $V_m = RP + 10$ mV. Channels open several times during a depolarization. Single openings (open circles) and openings in bursts (fitted circles) can also be observed. With chloramine-T the probability of finding openings at later times is significantly smaller than with ATX-II or with SCT. This can also be seen in Fig. 2, in which the averaged single-channel currents are compared. The largest noninactivating, steady-state current could be obtained with SCT, the smallest with CHL-T. The concentrations used (*see* Fig. 2 caption) resulted in maximum inhibition of the macroscopic inactivation. The integrals of the normalized averaged currents were (in arbitrary units) 1.0, 4.2, 6.7 and 10.6 for control, CHL-T, ATX-II and SCT, respectively.

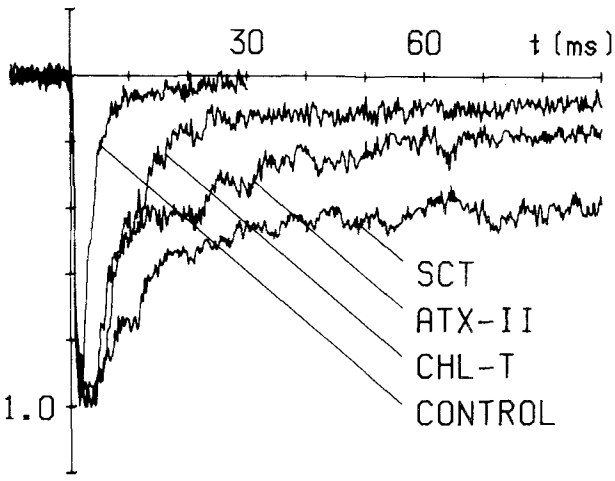


Fig. 2. Averaged single-channel current records obtained with unmodified (control) and with modified sodium channels as indicated. $V_H = RP - 60$ mV, $V_m = RP + 20$ mV; for the control patch $V_H = RP - 50$ mV, $V_m = RP + 15$ mV, $T = 12$°C. Concentrations of the substances are: CHL-T 0.5 mM, ATX-II 6.1 μM and SCT 220 nM. The currents are normalized to the peak value for better comparison

Single-Channel Current Amplitudes

For the three substances, a similar mean single sodium-channel current size of ~1.3 pA was measured at the same relative membrane potential of $V_m = RP$ mV with 160 mM external NaCl at 14 °C. The single-channel conductance of the SCT modified channels was linear measured between $RP - 20$ mV and $RP + 40$ mV and was 12.9 ± 2.7 pS ($n = 4$) at 12°C. The γ for CHL-T was 13.8 pS, for ATX-II 10.8 pS (calculated with $Q_{10} = 1.28$ from Nagy, 1987*b*). Similarly, 11.5 pS unitary conductance was reported for the ATX-II modified cardiac sodium channels (Schreibmayer et al., 1987). Subconductance current levels could occasionally be observed not only with the CHL-T and ATX-II modified channels, as previously described (Nagy, 1987*d*), but also with the SCT modified channels. They are demonstrated in Fig. 3*A*. Besides the most frequent

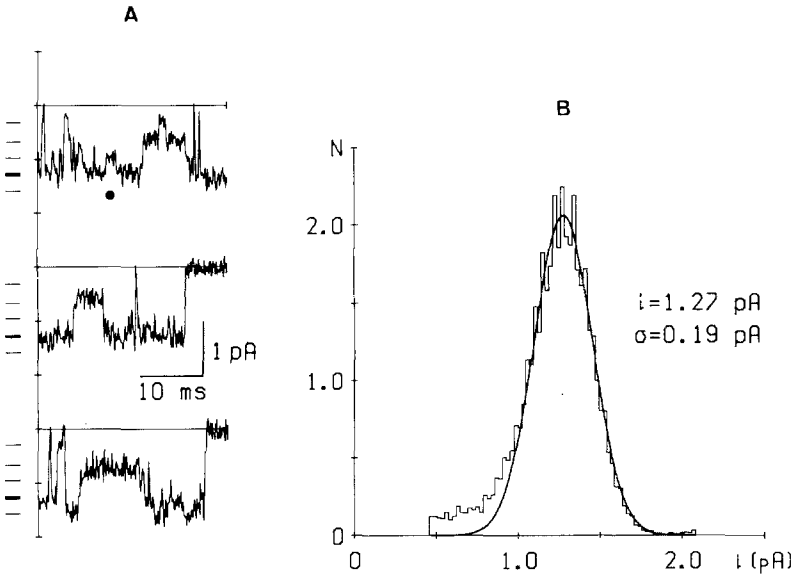


Fig. 3. (*A*) Subconductance levels of scorpion toxin modified sodium channels. (*B*) Current-amplitude histogram from the same patch. Segments (30 msec long) of current records are plotted in (*A*) demonstrating five different current levels. Horizontal bars on the left indicate the mean values for the sublevels (thin lines) and for the main current level (thick line) calculated on selected current records. The mean values are (in pA): 0.30, 0.67, 0.93, 1.27 and 1.57. Filled circle below the topmost record indicates a sublevel of 73% of the usual current size. The open-channel current-amplitude histogram in (*B*) was fitted by a single Gaussian function with parameters as indicated. The closed-channel current noise had a σ of 0.09 pA. *A* and *B* are from the same experiment as Fig. 1

current amplitude of 1.27 pA (estimated from the midpoint of the bell-shaped amplitude histogram, Fig. 3*B*), four different current levels can be seen. Mean values of the sublevels were calculated by averaging the digitized current points for sublevels longer that 0.56 msec (*see* Nagy, 1987*d*) on selected current records. The averages were (in % of the usual current size ± SD): 22.3 ± 4.7, 52.6 ± 4.2, 100.0 ± 11.4 and 124.1 ± 7.1. A sublevel having 73.4% of the most frequent current size (*see* filled circle in the topmost record in Fig. 3*A*) could be observed clearly only in <1% of openings of the selected records. This current level was suspected already for the CHL-T and ATX-II modified channels (Nagy, 1987*d*).

The sublevels cannot be recognized in the amplitude histogram (Fig. 3*B*) due to their relative short lifetime and rare occurrence. Only the deviation from the Gaussian fit indicates the presence of current levels smaller than the usual size. The σ of the Gaussian fit in Fig. 3*B* is 0.19 pA, which is about twice the closed-channel current noise of σ = 0.09 pA. This large SD of the main current size and the "unstable" open-channel current records (*see* record 5 and 6 for SCT in Fig. 1 and record 2 in Fig. 3*A*) may, partially, be caused by unresolved transitions between the sublevels of 73 and 124% and the main current level.

Single-Channel Closed and Open Times

Single-channel open and burst times were measured in patches containing 2 to 4 channels with the half-amplitude threshold criterion on records without superimposed openings. The burst time is the time of subsequent openings, which are separated by closed gaps shorter than a characteristic value t_c. t_c was determined from closed-time histograms fitted by the sum of two exponentials (Fig. 4; *see* Eqs. (1) and (2) in Materials and Methods). It is supposed that openings separated by closed gaps longer than t_c originate from different channels and openings separated by gaps shorter than t_c are from the reopening of the same channel. Examples of closed-time histograms and their fits by the sum of two exponentials are shown in Fig. 4 for the three substances. The characteristic time t_c varied from patch to patch for each substance.

It might be supposed that single openings (separated by closed gaps $>t_c$) and openings that appear in bursts (*see* open and filled circles in Fig. 1) originate from different channel types or conformations. Therefore, the open times for these two types of openings were calculated separately. Fig. 5 shows open-time histograms and their fits for single openings (upper plots) and for openings in bursts (lower plots) for CHL-T, ATX-II and SCT modified chan-

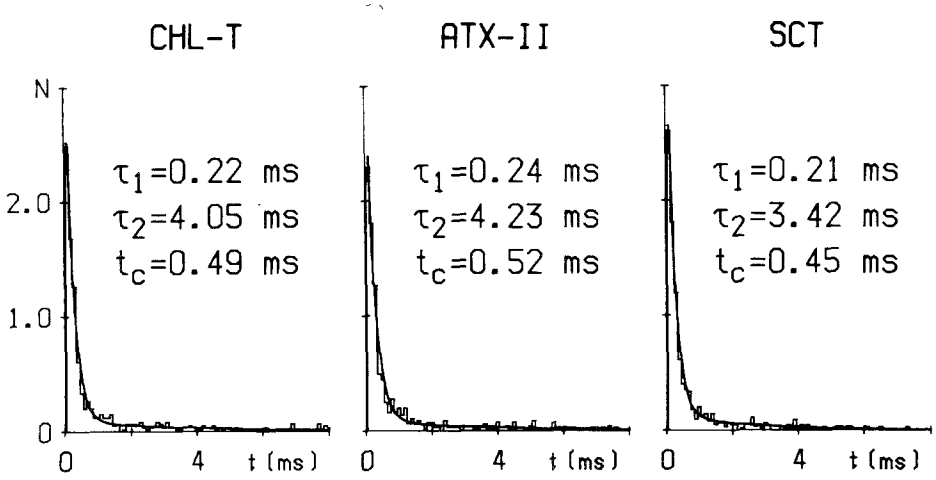


Fig. 4. Closed-time histograms constructed for chloramine-T (*CHL-T*), sea anemone (*ATX-II*) and scorpion toxin (*SCT*) modified sodium channels. The histograms were fitted by the sum of two exponentials resulting in time constants as indicated. From the time constants the characteristic time t_c was calculated by Eq. (2), as described in Materials and Methods

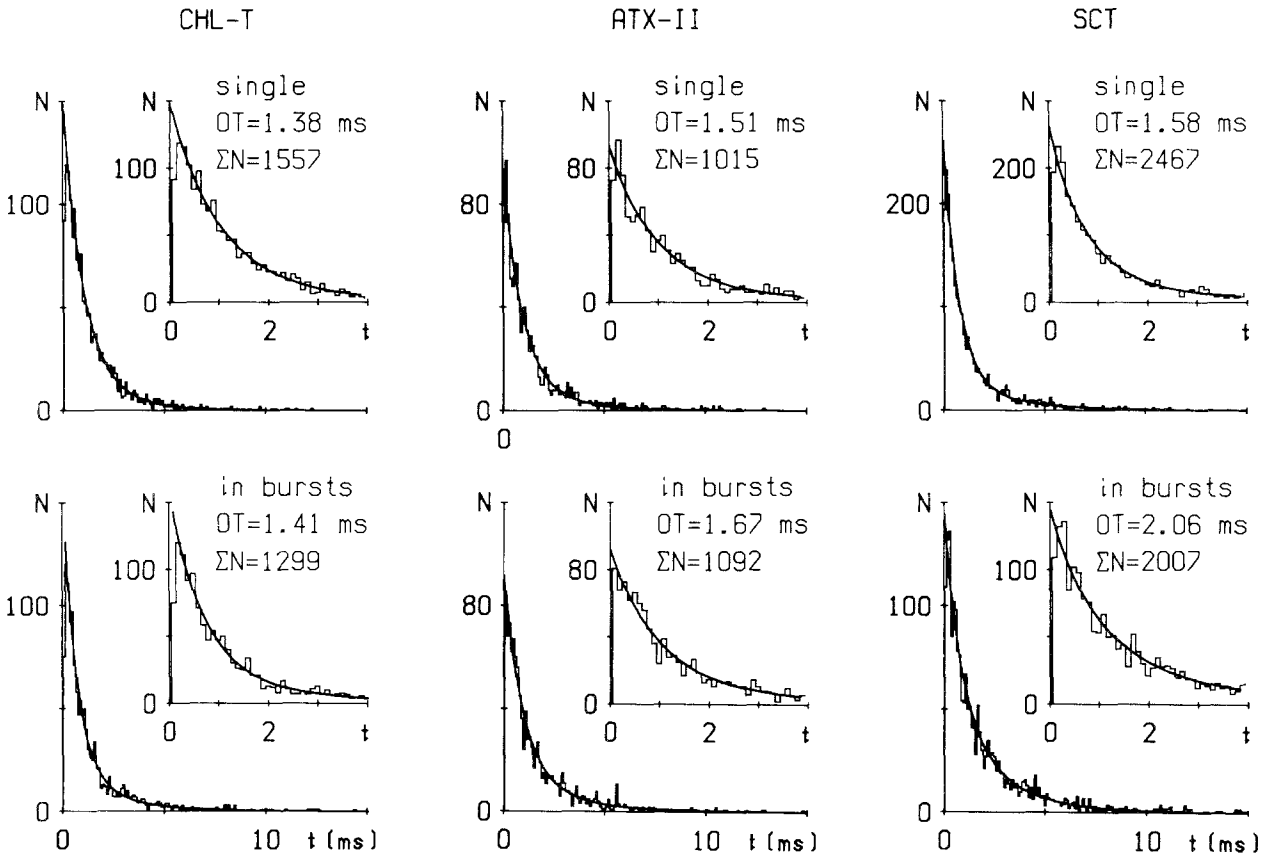


Fig. 5. Open-time histograms for CHL-T, ATX-II and SCT modified sodium channels. The upper histograms are constructed from openings that were separated by closed gaps $>t_c$ (single openings); the lower histograms are from openings in bursts with closed gaps $<t_c$ between the openings. Insets show the 0–4 msec intervals of the histograms on expanded time scale. Mean open times (*OT*) and the number of events (ΣN) are indicated. The histograms were fitted by the sum of two exponentials. Parameters of the fits are listed in Table 1 ($V_m = RP + 20$ mV, $T = 12$°C)

Table 1. Parameters obtained by fitting the sum of two exponentials, Eq. (1), to the open-time histograms in Fig. 5

	$\tau_1 \pm$ SEE (msec)	$\tau_2 \pm$ SEE (msec)	$w_1 \pm$ SEE	$w_2 \pm$ SEE	x^2(df)	P
CHL-T single	1.00 ± 0.05	2.77 ± 0.14	0.83 ± 0.12	0.17 ± 0.09	22.86(33)	0.91
in bursts	0.70 ± 0.04	2.62 ± 0.12	0.69 ± 0.05	0.31 ± 0.06	34.66(37)	0.58
ATX-II single	0.97 ± 0.04	3.38 ± 0.30	0.75 ± 0.08	0.25 ± 0.11	22.66(26)	0.65
in bursts	0.97 ± 0.09	3.23 ± 0.21	0.68 ± 0.06	0.32 ± 0.07	25.41(31)	0.75
SCT single	0.74 ± 0.04	3.24 ± 0.20	0.66 ± 0.03	0.34 ± 0.03	42.87(41)	0.39
in bursts	0.83 ± 0.07	2.75 ± 0.22	0.36 ± 0.04	0.64 ± 0.05	48.90(46)	0.36

Abbreviations: τ_i: time constant; SEE: standard error of the estimated value; w_i: weighting factor.

nels. The mean open times (OT) for the single and burst openings are similar for the CHL-T modified channels, but the OT is somewhat longer in bursts for the ATX-II and SCT modified channels. However, the Mann-Whitney-Wilcoxon test (Graf, Henning & Stange, 1966) indicated no significant difference between the open-time histograms of the single and the burst openings ($\alpha > 0.05$; two-tailed test).

The open-time histograms of both the single and burst openings could be best fitted by the sum of two exponentials. The parameters of the fits (*see* Table 1) indicate moderate differences between the single and burst openings. The weighting factors of the fast components (w_1) are reduced for the burst openings, and; therefore, the weighting factors of the slow components (w_2) increased, resulting in longer mean open times for the ATX-II and SCT modified channels (*see* OTs in Fig. 5). The fast time constant τ_1 for the burst openings is somewhat smaller than for single openings in CHL-T modified channels; therefore, the mean OT (Fig. 5) did not change considerably. τ_2 of the CHL-T and τ_1 and τ_2 of the ATX-II and SCT modified channels are similar for the single and burst openings. These similarities indicate that it is the same population of sodium channels that produces the single openings and the burst openings.

If single openings indicated toxin-free and bursts toxin-bound channels, the time constants of the first delay histograms constructed from single and burst openings could be different. However, both the fast and the slow time constants of these plots (*not shown*) were similar, suggesting also a homogeneous population of channels. This observation shows that the substances used do not cause a large shift of the activation parameters; therefore, a comparison of different patches on the basis of the first latency densities is feasible (*see* Materials and Methods).

The ratios of the number of events for single openings and openings in bursts were 1.20, 0.93 and 1.23 for the CHL-T, ATX-II and SCT modified channels, respectively (*see* Fig. 5). Thus, the tendency of channels to open in bursts is similar for the three substances at the applied concentrations. Therefore, the larger integral of the averaged current (Fig. 2) for ATX-II and SCT, compared to CHL-T, cannot be explained by a larger portion of the burst openings.

Burst Durations and Open Times During a Burst

Histograms of burst durations and of the total open time per bursts were constructed for consecutive openings that were separated by closed gaps $<t_c$. Parameters were calculated separately from bursts consisting of more than one opening, two openings and three openings. Burst-duration histograms consisting of more than one opening are shown in Fig. 6 (upper plots). The mean values indicate that SCT elicits the longest (6.14 msec) and CHL-T the shortest mean burst time (4.93 msec) as expected from the averaged single-channel currents (*see* Fig. 2). The mean number of openings during a burst (= mean burst time/mean open time in burst; from Figs. 5 and 6) is 3.5, 3.5 and 3.0 for CHL-T, ATX-II and SCT, respectively.

The lower plots in Fig. 6 demonstrate the distribution of the number of closed gaps per burst n. These distributions were best fitted by the sum of two geometric functions (*see* Eq. (4) in Materials and Methods). The fits resulted in two mean values (μ_1 and μ_2), the smaller about 1.4, the larger about 3.0. The mean values are similar for the three substances. The weight of the second component (a_2) was clearly larger for ATX-II than for SCT or CHL-T. a_2 for CHL-T was the smallest (0.11) as expected from the smallest mean burst time. The two mean values of the number of closings per burst distributions suggest that the modified channels may have two types of bursting mode; in one mode

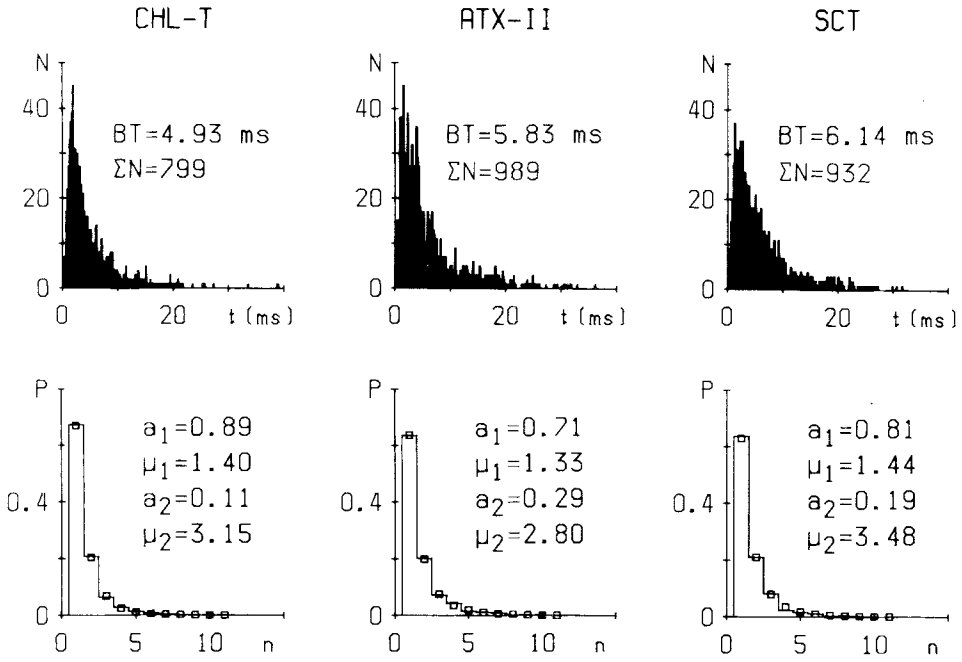


Fig. 6. Burst-duration histograms (upper plots) and the distributions of the number of closings per burst (n, lower plots) for the modified channels as indicated. Plots are from the same patches as Fig. 5. For the burst-duration histograms, the mean burst time (BT) and the number of events (ΣN) are given. The distributions of closings per burst were fitted by a geometric function; *see* Eq. (4) in Materials and Methods. The weighting factors a_1, a_2 and the mean values of the components μ_1, μ_2 are indicated in the plots

the channels open 2–3 times, in the other mode (observed with lower probability) 4–5 times. (Note that the number of openings in a burst equals the number of closings plus one.)

One possibility to investigate whether bursts consist of the same sort of opening of a channel is to construct burst open-time histograms (e.g., the total open time per burst) from two, three, etc., openings and to fit the histograms with the gamma function (Colquhoun & Sakmann, 1985). Similar histograms indicate that the bursts are due to the same channel (having a single open time) or due to the same open conformation of a channel (having multiple open states). (For the gamma function, it is supposed that the components have an exponential distribution. As the open-time distributions in the present case were fitted by the sum of two exponentials, the time constant τ of the gamma function indicates a mean value of the open time.)

The burst open-time histograms for two and three openings and their fits with the gamma function, Eq. (3), are shown in Fig. 7. For CHL-T, the time constants (τ) of the fits are similar for bursts with three openings ($k = 3$) and for bursts with two openings ($k = 2$). For ATX-II and SCT, the time constants are larger for $k = 3$ than for $k = 2$. The different time constants for two and three openings may indicate that the open time of the consecutive openings is different, probably due to different open states of a channel. For this investigation another approach was followed; open-time histograms were constructed from the first, second and third openings of a burst separately. These histograms are shown in Fig. 8 for CHL-T, ATX-II and SCT modified channels. The mean open times (OT_i) indicate moderate changes for the consecutive openings; however, the biexponential fits revealed some differences. (Note that due to the biexponential fits the mean open times should not necessarily be different for different histograms.)

Open-time histograms of the first, second and third openings of bursts (Fig. 8) could be best fitted by the sum of two exponentials. The parameters are listed in Table 2. For each substance, the weighting factor of the first component, w_1, is smaller for the first than for the second or the third openings. Both the fast and the slow time constants increase with the order of openings. However, significant difference between the histograms could not be obtained systematically. In one-third of the experiments and for the example shown in Fig. 8, the open-time histograms of the first and the third openings were significantly different ($\alpha \leq 0.05$, two-tailed Mann-Whitney-Wilcoxon test; Graf et al., 1966). In a few cases, a significant difference could also be found between the histograms of the first and the second and the second and the third openings at $\alpha \leq 0.05$. On the basis of these statistical tests and the non-

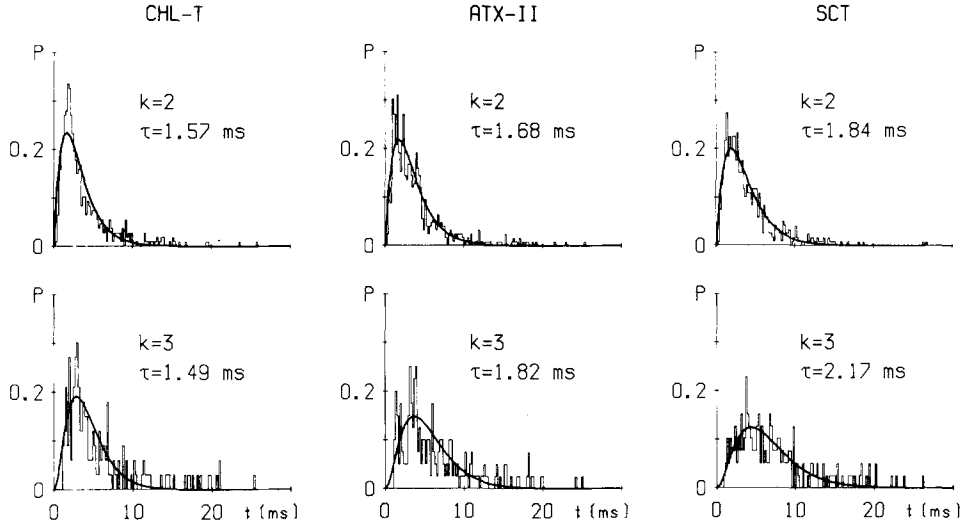


Fig. 7. Histograms of the open times during bursts for the modified channels constructed from bursts consisting of two openings ($k = 2$, upper plots) or three openings ($k = 3$, lower plots). The distributions were fitted by the gamma function given by Eq. (3) in Materials and Methods. The estimated parameters and their SE are (in msec): for CHL-T 1.57 ± 0.02 ($k = 2$) and 1.49 ± 0.04 ($k = 3$), for ATX-II 1.68 ± 0.04 ($k = 2$) and 1.82 ± 0.06 ($k = 3$) and for SCT 1.84 ± 0.03 ($k = 2$) and 2.17 ± 0.06 ($k = 3$)

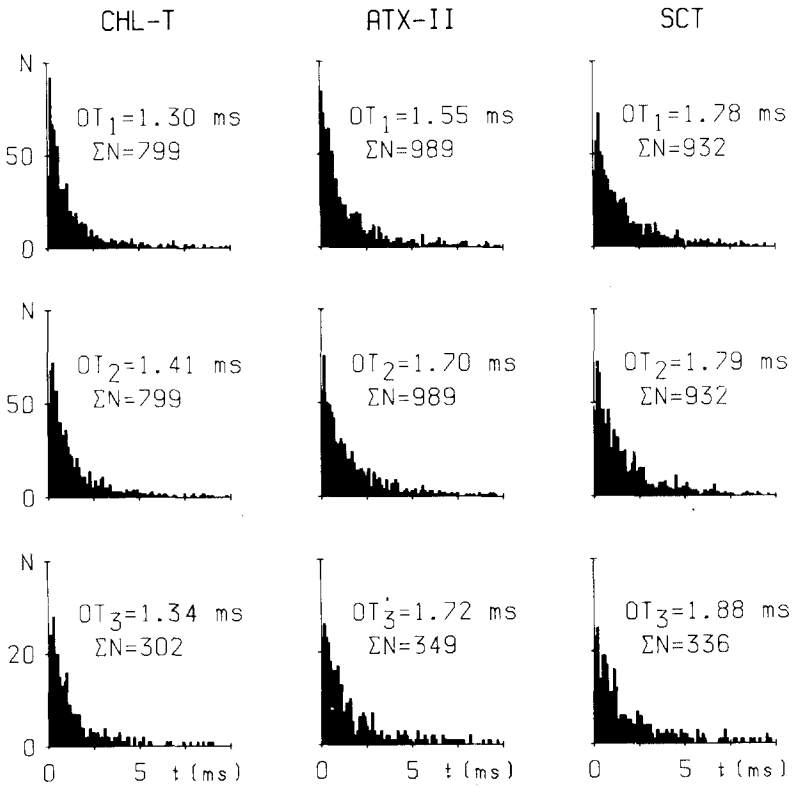


Fig. 8. Open-time histograms for the modified channels constructed from the first (upper plots), the second (middle plots) and the third openings of a burst (lower plots). OT_1, OT_2 and OT_3 are the mean open times of the first, second and third opening, respectively. ΣN are the number of events. Plots are from the same experiments as Figs. 5, 6 and 7. The histograms were fitted by the sum of two exponentials; *See* Eq. (1) in Materials and Methods. The parameters of the fits are listed in Table 2

systematic differences, it cannot be strictly stated that the consecutive openings of bursts are different. However, the examples of significant differences, the different time constants of the gamma function for $k = 2$ and $k = 3$ with ATX-II and SCT (Fig. 7) and the increasing tendency of the time con-

Table 2. Parameters of the exponential fits for Fig. 8

		$\tau_1 \pm$ SEE (msec)	$\tau_2 \pm$ SEE (msec)	$w_1 \pm$ SEE	$w_2 \pm$ SEE	x^2(df)	P
CHL-T	1st open	0.32 ± 0.07	1.25 ± 0.16	0.29 ± 0.12	0.71 ± 0.15	21.18(24)	0.63
	2nd open	0.76 ± 0.09	3.00 ± 0.49	0.71 ± 0.12	0.29 ± 0.14	20.20(20)	0.45
	3rd open	0.90 ± 0.11	5.69 ± 1.04	0.79 ± 0.06	0.21 ± 0.06	8.74(15)	0.89
ATX-II	1st open	0.64 ± 0.06	2.42 ± 0.27	0.49 ± 0.10	0.51 ± 0.11	19.39(27)	0.85
	2nd open	0.78 ± 0.07	2.42 ± 0.39	0.58 ± 0.15	0.42 ± 0.10	25.73(31)	0.73
	3rd open	1.03 ± 0.10	7.02 ± 1.33	0.74 ± 0.05	0.26 ± 0.06	6.68(15)	0.96
SCT	1st open	0.56 ± 0.09	2.24 ± 0.40	0.30 ± 0.08	0.70 ± 0.10	24.96(38)	0.95
	2nd open	1.02 ± 0.11	3.09 ± 0.25	0.59 ± 0.22	0.41 ± 0.20	24.08(25)	0.51
	3rd open	0.95 ± 0.09	3.59 ± 0.51	0.56 ± 0.15	0.44 ± 0.17	12.15(18)	0.84

Abbreviations as in Table 1.

Table 3. Spearman's correlation coefficients for consecutive openings and closings

		r	u	α	N
CHL-T	O_1-O_2	+0.21	5.80	$<10^{-4}$	735
	O_2-O_3	+0.20	3.00	2.7×10^{-3}	240
	O_1-C_1	−0.01	0.23	0.81	735
	O_2-C_2	−0.07	1.11	0.26	240
	C_1-O_2	−0.03	0.83	0.40	735
	C_2-O_3	−0.05	0.78	0.43	240
ATX-II	O_1-O_2	+0.21	6.22	$<10^{-4}$	884
	O_2-O_3	+0.25	4.36	$<10^{-4}$	310
	O_1-C_1	−0.10	2.97	2.8×10^{-3}	884
	O_2-C_2	−0.18	3.13	2.1×10^{-3}	310
	C_1-O_2	−0.09	2.54	0.01	884
	C_2-O_3	−0.16	2.72	6.6×10^{-3}	310
SCT	O_1-O_2	+0.20	5.96	$<10^{-4}$	847
	O_2-O_3	+0.19	3.06	2.0×10^{-3}	269
	O_1-C_1	−0.05	1.52	0.12	847
	O_2-C_2	−0.11	1.80	0.07	269
	C_1-O_2	−0.06	1.84	0.06	847
	C_2-O_3	−0.10	1.67	0.09	269

Abbreviations: r: correlation coefficient, u: critical value, α: significance level, N: number of event pairs.

stants with the order number (Table 2) suggest that under certain conditions different open states of channels can be observed during bursts.

Correlation Between Open and Closed Times of Bursts

Correlation between the subsequent open and closed times can give information about the transition pathways that link the states. As patches contained more than one channel, calculations of correlation must be restricted to the open and closed times during bursts, where the openings are due to the same channel.

For the three substances, correlations were calculated for the following open and closed times; first and second openings (O_1-O_2), second and third openings (O_2-O_3), first openings and first closings (O_1-C_1), second openings and second closings (O_2-C_2), first closing and second openings (C_1-O_2) as well as second closing and third openings (C_2-O_3). Spearman's rankcorrelation coefficients r (Graf et al., 1966), critical values u, significance levels α and the number of event pairs N are shown in Table 3. A significant positive correlation was found between the O_1-O_2 and the O_2-O_3 openings for each substance. No correlation was found for any open and closed intervals with CHL-T and SCT. For ATX, the correlations for open and closed intervals are negative and highly significant.

Discussion

The main goal of this paper was to describe: (i) the single-channel conductances and (ii) the kinetic properties of the voltage-gated sodium channels after modification by chloramine-T, sea anemone toxin and scorpion toxin.

Single-Channel Conductances

No significant difference was observed for the single-channel conductance measured with the three substances in the potential range of $RP - 20$ mV and $RP + 40$ mV. It was linear and was about 13 pS at 12°C, which is very similar to the conductance of the unmodified channels (Nagy et al., 1983) and to that of the CHL-T and ATX-II modified channels (Nagy, 1987*d*). Sublevels of about 22, 53, 73 and 124% of the usual current size were observed for the SCT modified channels (Fig. 3). The 73% level was not observed for the CHL-T and ATX-II modified channels, but the other levels were similar to those reported for the CHL-T and ATX-II modified

channels (Nagy, 1987*d*). It seems likely that unmodified channels have also different open conformations with different conductivity.

Subconductance states for batrachotoxin-modified sodium channels incorporated in planar lipid bilayers have been observed. Urban et al. (1987) reported two current levels for sodium channels from eel electroplax and dog brain with conductances of 50% and about 35% of the fully open state. Green, Weiss and Andersen (1987) observed two subconductances on sodium channels from canine forebrain. These observations also support the idea that native channels have different subconductance states, which due to their short lifetimes and rare occurrences, are overlooked.

Kinetic Properties of the Modified Channels

Single-channel current records, in the presence of each substance, showed consecutive opening and closing. This kind of opening was also observed on pronase-modified channels in dorsal root ganglion neurons (Carbone & Lux, 1986) and on papain-modified channels in neuroblastoma cells (Quandt, 1987), but never observed on native channels in neuroblastoma cells (Aldrich, Corey & Stevens, 1983; Nagy et al., 1983; Nagy, 1987*b*,*d*; Quandt, 1987) or channels modified by batrachotoxin (Quandt & Narahashi, 1982) or delta-methrin (Chinn & Narahashi, 1986). Bursting behavior and variable open time with single openings lasting 70 msec were reported for the sodium channels in neuroblastoma cells after modification by CHL-T (McCarthy & Yeh, 1987). I have never observed such long openings. The discrepancy could be explained by the different patch configuration, the different temperature and perhaps a different filter frequency; McCarthy and Yeh (1987) worked with excised patches at 10°C, whereas I used cell-attached patches at $T \geq 12$°C. ATX-II modified cardiac sodium channels also show bursting and an increased open time (Schreibmayer et al., 1987).

The mean open time of the CHL-T, ATX-II and SCT modified channels was about 1.4, 1.6 and 1.8 msec, respectively. These values are 31, 50 and 68% larger than the mean open time of the native channels, which is 1.07 msec at 12°C (calculated from Nagy, 1987*a*, with a Q_{10} of 2.2). These changes are significantly smaller than those reported for ATX-II on cardiac cells (Schreibmayer et al., 1987), for papain (Quandt, 1987) and batrachotoxin on neuroblastoma cells (Quandt & Narahashi, 1982) and for N-bromoacetamide on rat myotubes (Patlak & Horn, 1982). The discrepancies could partly be explained by the different filter frequency and potential range used in different laboratories. The mean open times of the modified channels described here are smaller than expected from the averaged current records (Fig. 2). Therefore, the increased integral of the averaged single-channel current records of the modified channels compared to that of the native channels is mainly due to the bursting behavior of the channels.

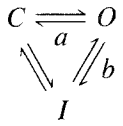
The open-time histograms of single and burst openings are similar (Fig. 5). It should be noted that CHL-T has an irreversible effect on the decay of the macroscopic current, whereas the effect of ATX-II and SCT are reversible. In the case of ATX-II and SCT, the openings in bursts might originate from channels to which a toxin is bound at the moment of the opening and the single openings may represent toxin-free channels. The two types of openings should therefore be different. However, the similar open-time histograms for bursts and single openings (Fig. 5 and Table 1) indicate that at the toxin concentrations used (which result in the largest inhibition of the macroscopic inactivation) only a small portion of channels is toxin-free. This is also suggested by the difference between the mean open time of the single openings and that of the native channels.

The biexponential fits of the open-time histograms support the idea of two open states of the modified channels, as also suggested for the native channels (Nagy et al., 1983; Nagy, 1987*a*) and for the papain-modified channels (Quandt, 1987). (A third time constant, which was ~0.4 msec at 8°C, (Nagy, 1987*a*) could not be identified in the present experiments at $T \geq 12$°C.) The similarity of the time constants of the open-time histograms for single and burst openings suggests that the two types of openings are due to the same channel type, which has kinetically distinct open states.

The mean burst durations in Fig. 6 were 4.93, 5.83 and 6.14 msec for CHL-T, ATX-II and SCT, respectively, corresponding to average values of 3.5, 3.5 and 3.0 openings per burst, respectively. These numbers are very similar and cannot explain the different mean currents in Fig. 2. The relative integrals of the mean currents for CHL-T, ATX-II and SCT are 4.2, 6.7 and 10.6. Dividing these values by the mean burst durations, the relative number of bursts per unit current integral is obtained as 0.85, 1.15 and 1.73. Therefore, the prolonged decay of the mean currents measured with CHL-T, ATX-II and SCT can be explained by a complex common effect of the substances on the channel open time, burst time and closed time.

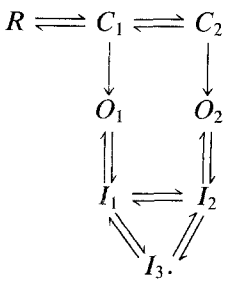
It can be supposed that the bursting of the modified channels indicates repetitive transitions be-

tween the open and the inactivated state. This hypothesis is supported by the following estimate. Consider the following general scheme



where a and b are the rate constants for the transition from the open (O) to the closed (C) and the transition from the open to the inactivated (I) state, respectively. For this scheme the channel open time is $OT = (a + b)^{-1}$ and the open-inactivated transition probability is $P_I = b/(a + b)$. Taking $P_I = 0.8$ (*i.e.*, $b = 4a$) for membrane potentials ≥ -20 mV (Aldrich et al., 1983; Horn, Vandenberg & Lange, 1984), and supposing that the substances inhibit the $O \rightarrow I$ transition, the largest 68% increase of the channel open time measured with SCT corresponds to a twofold decrease of the rate constant b. (The open-resting and inactivated-open rate constants are supposed to remain unchanged.) This new open-inactivated rate constant ($b = 2a$) results in $P_I = 0.67$, which corresponds to about a 13-mV negative shift on the voltage axis (calculated from the $P_I(V)$ relationship of Horn et al., 1984). Therefore, if the studied substances caused only a moderate decrease in the open-inactivated rate constant, the modified channels should behave as the native channels at a 13 mV more negative potential. Instead, the modified channels displayed bursting, which was never observed with native channels in neuroblastoma cells. This suggests that the flickering occurs mainly between the open and the inactivated state and that the activation pathway is nearly irreversible as suggested by Gonoi and Hille (1987). It follows that the absorbing property of the inactivated state is strongly reduced (*i.e.*, the inactivated-open probability is increased) by the tested substances. Note that the shift estimated above is very close to the 7–10 mV negative shift reported for the sodium current modified by scorpion toxin and coral toxin in neuroblastoma cells $N18$ (Gonoi & Hille, 1987).

The above conclusions are summarized in the following tentative scheme



Here R, C_i, O_i and I_i represent the resting, closed, open and inactivated states, respectively. This scheme is similar to the one presented previously (Nagy, 1987*a*), but assumes different inactivated states. Justification of this assumption comes from Table 3. A positive correlation was found between the first and the second as well as the second and the third openings of bursts for each modified channel (Table 3). This positive correlation suggests that during bursting the channel opens to the same open state (Colquhoun & Hawkes, 1987; Jackson et al., 1983). (This conclusion confirms the previous supposition that openings that are separated by closed gaps $<t_c$ are due to the same channel.) The positive correlation between the successive open times indicates that the transition probability from the closed state (or in the present case from the inactivated state) to the adjacent closed (or inactivated) state is smaller than the probability of revisting the previous open state.

A negative correlation was observed between the closed and open lifetimes with ATX-II and SCT, but not with CHL-T (Table 3). These inverse relations indicate that an open state with a shorter lifetime is adjacent to a closed (or inactivated) state with a longer lifetime. Negative correlation was also observed for the open and closed intervals (McManus, Blatz & Magleby, 1985). The finding that C_i-O_i does not correlate for CHL-T indicates that substances modify the transition pathways differently and a uniform description cannot be given.

As the mean number of openings in bursts (on average 3.5 for CHL-T and ATX-II, and 3.0 for SCT) does not increase proportionally to the open-channel probability (Fig. 2), it can be supposed that the same channel reopens from the inactivated state or flickers between the open and inactivated state after a longer stay ($>t_c$) in an inactivated state. Therefore, the existence of a third inactivated state (I_3) has been postulated. Channels stay longer in I_3 than in I_1 or I_2 and can return (through I_1 or I_2) to any of the open states. However, as mentioned, due to the multichannel patches, this question could not be further studied. I_3 might be identical with the "hibernating state" suggested by Horn et al., (1984). It is still not clear whether the multiple inactivated states are always present (but only observable with substances that inhibit the inactivation) or elicited by these substances.

I thank Professor H. Meves for support, helpful discussions and comments on the manuscript. Thanks to Dr. D. Hof for the fits of Fig. 7, Dr. T.D. Plant for reading the manuscript and Mrs. R. Stolz for typing it. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 246).

References

Aldrich, R.W., Corey, D.P., Stevens, C.F. 1983. A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature (London)* **306:**436–441

Bergman, C., Dubois, J.M., Rojas, E., Rathmayer, W. 1976. Decreased rate of sodium conductance inactivation in the node of Ranvier induced by polypeptide toxin from sea anemone. *Biochim. Biophys. Acta* **455:**173–184

Carbone, E., Lux, H.D. 1986. Sodium channels in cultured chick dorsal root ganglion neurons. *Eur. Biophys. J.* **13:**256–271

Chinn, K., Narahashi, T. 1986. Stabilization of sodium channel states by deltamethrin in mouse neuroblastoma cells. *J. Physiol. (London)* **380:**191–207

Colquhoun, D., Hawkes, A.G. 1987. A note on correlation in single ion channel records. *Proc. R. Soc. London B* **230:**15–52

Colquhoun, D., Sakmann, B. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle endplate. *J. Physiol. (London)* **369:**501–557

Colquhoun, D., Sigworth, F.J. 1983. Fitting and statistical analysis of single-channel records. *In:* Single Channel Recording. B. Sakmann and E. Nehr, editor. Plenum, New York, pp. 191–263

Drews, G. 1987. Effects of chloramine-T on charge movement and fraction of open channels in frog nodes of Ranvier. *Pfluegers Arch.* **409:**251–257

Gonoi, T., Hille, B. 1987. Gating of Na channels: Inactivation modifiers discriminate among models. *J. Gen. Physiol.* **89:**253–274

Graf, U., Henning, H-J., Stange, K. 1966. Formeln und Tabellen der mathematischen Statistik. Springer, Berlin—Heidelberg—New York

Green, W.N., Weiss, L.B., Andersen, O.S. 1987. Batrachotoxin-modified sodium channels in planar lipid bilayers: Ion permeation and block. *J. Gen. Physiol.* **89:**841–872

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hof, D. 1986. A pulse generating and data recording system based on the microcomputer PDP 11/23. *Comput. Method. Program. Biomed.* **23:**309–315

Horn, R., Vandenberg, C.A., Lange, K. 1984. Statistical analysis of single sodium channels: Effect of N-bromoacetamide. *Biophys. J.* **45:**323–335

Jackson, M.B., Wong, B.S., Morris, C.E., Lecar, H. 1983. Successive openings of the same acetylcholine receptor channel are correlated in open time. *Biophys. J.* **42:**109–114

Koppenhofer, E., Schmidt, H. 1968. Die Wirkung von Skorpiongift auf die Ionenströme des Ranvierschen Schnürringes. II. Unvollständige Natrium Inaktivierung. *Pfluegers Arch.* **303:**150–161

McCarthy, W.A., Yeh, J.Z. 1987. Chloramine-T removes closed and open Na-channel inactivation in N1E-115 neuroblastoma cells: A single channel/multi-channel-patch study. *Biophys. J.* **51:**436a

McManus, O.B., Blatz, A.L., Magleby, K.L. 1985. Inverse relationship of the durations of adjacent open and shut intervals for Cl and K channels. *Nature (London)* **317:**625–627

Moolenaar, W.H., Spector, I. 1978. Ionic currents in cultured mouse neuroblastoma cells under voltage-clamp conditions. *J. Physiol. (London)* **278:**265–286

Meves, H., Rubly, N. 1986. Kinetics of the sodium current and gating current in the frog node of Ranvier. *Pfluegers Arch.* **407:**18–26

Meves, H., Rubly, N., Watt, D.D. 1982. Effect of toxins isolated from the venom of the scorpion *Centruroides sculpturatus* on the Na currents of the node of Ranvier. *Pfluegers Arch.* **393:**56–62

Meves, H., Simard, J.M., Watt, D.D. 1984. Biochemical and electrophysiological characteristics of toxins isolated from the venom of the scorpion *Centruroides sculpturatus*. *J. Physiol. (Paris)* **79:**185–191

Nagy, K. 1987*a*. Evidence for multiple open states of sodium channels in neuroblastoma cells. *J. Membrane Biol.* **96:**251–262

Nagy, K. 1987*b*. Kinetic behavior of chloramine-T and sea anemone toxin modified channels in neuroblastoma cells. *Pfluegers Arch.* **408:**R37

Nagy, K. 1987*c*. Single sodium channel currents on mouse neuroblastoma cells after pharmacological inhibition of inactivation. *9th Int. Biophys. Congr. Abstr.* p. 194

Nagy, K. 1987*d*. Subconductance states of single sodium channels modified by chloramine-T and sea anemone toxin in neuroblastoma cells. *Eur. Biophys. J.* **15:**129–132

Nagy, K., Kiss, T., Hof, D. 1983. Single Na channels in mouse neuroblastoma cell membrane: Indications for two open states. *Pfluegers Arch.* **399:**302–308

Neumcke, B., Schwarz, W., Stampfli, R. 1985. Comparison of the effects of Anemonia toxin II on sodium and gating currents in frog myelinated nerve. *Biochim. Biophys. Acta* **814:**111–119

Patlak, J.B., Horn, R. 1982. Effect of N-bromoacetamide on single sodium channel currents in excised membrane patches. *J. Gen. Physiol.* **79:**333–351

Quandt, F. 1987. Burst kinetics of sodium channels which lack fast inactivation in mouse neuroblastoma cells. *J. Physiol. (London)* **392:**563–585

Quandt, F.N., Narahashi, T. 1982. Modification of single Na^+ channels by batrachotoxin. *Proc. Natl. Acad. Sci. USA* **79:**6732–6736

Rack, M., Rubly, N., Waschow, C. 1986. Effects of some chemical reagents on sodium current inactivation in myelinated nerve fibers of the frog. *Biophys. J.* **50:**557–564

Schreibmayer, W., Kazerani, H., Tritthart, H.A. 1987. A mechanistic interpretation of the action of toxin II from *Anemonia sulcata* on the cardiac sodium channel. *Biochim. Biophys. Acta* **901:**273–282

Strichartz, G., Rando, T., Wang, G.K. 1987. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **10:**237–267

Ulbricht, W., Schmidtmayer, J. 1981. Modification of sodium channels in myelinated nerve by *Anemonia sulcata* toxin II. *J. Physiol. (Paris)* **77:**1103–1111

Urban, B.W., Recio-Pinto, E., Duch, D.S., Paranicas, M. 1987. Several conductance levels in steady-state sodium channels. *Pfluegers Arch. Eur. J. Physiol.* **408:**R31

Wang, G.K. 1984. Irreversible modification of sodium channel inactivation in toad myelinated nerve fibres by the oxidant chloramine-T. *J. Physiol. (London)* **346:**127–141

Wang, G.K., Strichartz, G. 1985. Kinetic analysis of the action of *Leiurus scorpion* α-toxin on ionic currents in myelinated nerve. *J. Gen. Physiol.* **86:**739–762

Wang, G.K., Brodwick, M.S., Eaton, D.C. 1985. Removal of sodium channel inactivation in squid axon by the oxidant chloramine-T. *J. Gen. Physiol.* **86:**289–302

Received 17 November 1987; revised 27 June 1988