Kinetics of 9-Aminoacridine Block of Single Na Channels

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ABSTRACT The kinetics of 9-aminoacridine (9-AA) block of single Na channels in neuroblastoma N1E-115 cells were studied using the gigohm seal, patch clamp technique, under the condition in which the Na current inactivation had been eliminated by treatment with *N*-bromoacetamide (NBA). Following NBA treatment, the current flowing through individual Na channels was manifested by square-wave open events lasting from several to tens of milliseconds. When 9-AA was applied to the cytoplasmic face of Na channels at concentrations ranging from 30 to 100 μM, it caused repetitive rapid transitions (flickering) between open and blocked states within single openings of Na channels, without affecting the amplitude of the single channel current. The histograms for the duration of blocked states and the histograms for the duration of open states could be fitted with a single-exponential function. The mean open time (τ_o) became shorter as the drug concentration was increased, while the mean blocked time (τ_b) was concentration independent. The association (blocking) rate constant, k, calculated from the slope of the curve relating the reciprocal mean open time to 9-AA concentration, showed little voltage dependence, the rate constant being on the order of 1×10^7 $M^{-1}s^{-1}$. The dissociation (unblocking) rate constant, l, calculated from the mean blocked time, was strongly voltage dependent, the mean rate constant being 214 s^{-1} at 0 mV and becoming larger as the membrane being hyperpolarized. The voltage dependence suggests that a first-order blocking site is located at least 63% of the way through the membrane field from the cytoplasmic surface. The equilibrium dissociation constant for 9-AA to block the Na channel, defined by the relation of l/k, was calculated to be 21 μM at 0 mV. Both τ_o^{-1} and τ_b^{-1} had a Q_{10} of 1.3, which suggests that binding reaction was diffusion controlled. The burst time in the presence of 9-AA, which is the sum of open times and blocked times, was longer than the lifetime of open channels in the absence of drug. All of the features of 9-AA block of single Na channels are compatible with the sequential model in which 9-AA molecules block open Na channels, and the blocked channels could not close until 9-AA molecules had left the blocking site in the channels.

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INTRODUCTION

9-Aminoacridine (9-AA) blocks Na channels in squid giant axons in a frequency- and voltage-dependent manner. The frequency-dependent block requires the presence of the inactivation of Na channels (Yeh, 1979). The voltage-dependent block has been thought to reflect plugging of the ion-conducting pathway by the drug molecule, which binds to a specific site within the Na channel in a manner dependent on membrane potential (Cahalan, 1978; Yeh, 1979). Many local anesthetics share this type of blocking action (Cahalan, 1978; Courtney, 1975; Khodorov et al., 1976; Hille, 1977; Schwarz et al., 1977; Lipicky et al., 1978). Some of these compounds also exert voltage-dependent blocking action on agonist-activated ionic channels (Steinbach, 1968*a*, *b*; Maeno et al., 1971; Kordas, 1970; Beam, 1976*a*, *b*; Ruff, 1977; Adams, 1976, 1977; Yamamoto and Washio, 1979). Previous studies with macroscopic ionic currents suggest that drug block of ionic channels could be explained by a sequential model. In this model, drug molecules are thought to bind to open Na channels, resulting in a total loss of channel conductance (channel occlusion), and the drug molecules have to be released from the channels before they can close. Lipicky et al. (1978) have proposed an alternative mechanism for yohimbine action. This mechanism does not call for channel occlusion by a drug molecule, but it requires changes in channel kinetics in the presence of drug (a modified kinetic model). Recently, Gilbert and Lipicky (1981) have extended this model as a plausible mechanism for local anesthetic action.

The sequential model for open channel blocking has been tested in various types of ionic channels with single channel recordings. By using the patch clamp, single channel recording technique (Neher and Sakmann, 1976), Neher and Steinbach (1978) first visualized the blocking reaction of local anesthetics on individual acetylcholine-activated ionic channels. They observed that in the presence of lidocaine derivative QX-222, a single long, square pulse caused by the opening and closing of acetylcholine (ACh) channels was chopped into bursts of much shorter pulses (flickering) (Neher and Steinbach, 1978). Later, Ogden et al. (1981) found that an uncharged anesthetic, benzocaine, caused a similar flickering of single ACh channel currents. In sarcoplasmic reticulum K^+ channels, *n*-alkyl-*bis*-α,ω-trimethylammonium compounds with a long carbon chain such as decamethonium also caused an open channel to flicker between fully conducting and nonconducting states (Coronado and Miller, 1980, 1982; Miller, 1982). The flickering within a burst has been interpreted as representing a blocking and unblocking of an open channel by the drug molecule.

Thus far, no studies have been done on the block of single Na channels by local anesthetics or related compounds, although ionic block of Na channels by tetramethylammonium (TMA) and Ca^{2+} has been reported (Horn et al., 1981; Yamamoto et al., 1984). Neither TMA nor Ca^{2+} induced flickering of the current; they simply reduced the single channel conductance in a voltage-dependent manner (Horn et al., 1981; Yamamoto et al., 1984). A direct demonstration of occlusion of single Na channels by a drug molecule that produces frequency-dependent block of macroscopic currents would provide the crucial evidence for the occlusion model.

This paper represents the first demonstration that Na channels flicker in the presence of an open channel blocker, 9-AA. In addition, we have determined the association and dissociation rates of 9-AA binding, as well as the electrical distance to the binding site from the cytoplasmic surface of the membrane, by directly measuring the blocking and unblocking reaction. The validity of the sequential model was confirmed on the basis of single channel measurements in the absence of channel inactivation. A preliminary account of this work has appeared (Quandt et al., 1982).

MATERIALS AND METHODS

All experiments were carried out with N1E-115 neuroblastoma cells. These cells were maintained in tissue culture and grown in Dulbecco's modified Eagle's medium, supplemented with 10% newborn calf serum at 37°C in humidified air containing 10% CO_2. 3 d to 2 wk before use, cells were grown on coverslips in media to which 2% dimethylsulfoxide (DMSO) had been added in order to enhance the expression of neuronal characteristics (Kimhi et al., 1976).

Single channel currents were recorded from excised membrane patches using the gigohm seal, patch clamp technique (Hamill et al., 1981). The cells were initially immersed in the normal saline containing 125 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 55 mM sucrose, and 20 mM HEPES, and the pH was adjusted to 7.3 with NaOH. The patch pipette was filled with the high Na external solution composed of 250 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 20 mM HEPES. The pH was adjusted to 7.3 with NaOH. After excision of a membrane patch (see below), the perfusate in the chamber was switched to the internal solution. The internal solution contained 150 mM CsF, 1 mM Na-HEPES, 20 mM HEPES, and 145 mM sucrose; the pH was adjusted to 7.2 with CsOH. All solutions were filtered immediately before use through a membrane filter with 0.45 μm pore size (Gelman Instrument Co., Bedford, MI).

The formation of a gigohm seal between the patch electrode and the cell membrane was initiated by lowering the pressure in the pipette. Inside-out membrane patches were obtained as follows. After establishing a gigohm seal, depolarizing steps of 60 mV in amplitude, superimposed on a hyperpolarizing holding potential of −30 to −40 mV (in addition to the cell resting membrane potential), were applied to the bath to monitor the activity of single Na channels in the membrane patch. When the single channel opening events had been established, the bathing solution was changed from the normal external solution to the internal solution. 3 min after switching the solution, the membrane patch was excised by sudden withdrawal of the electrode from the cell.

To remove Na inactivation (Hodgkin and Huxley, 1952), the bathing solution was further switched to an internal solution containing 300 μM *N*-bromoacetamide (NBA), which is known to eliminate the inactivation of the Na current (Oxford et al., 1978; Patlak and Horn, 1982). NBA was then washed out with the NBA-free internal solution to prevent deterioration of the membrane. 9-AA was always added to the internal solution when indicated.

The holding and command voltages generated by a computer were applied to the bath via an Ag-AgCl pellet. The interior of the pipette was kept at virtual ground and the bath potential was measured using a separate reference microelectrode filled with 3 M CsCl. To measure membrane currents, an operational amplifier (3523; Burr-Brown Research Corp., Tucson, AZ) was used as the current-to-voltage converter with a feedback resistor of 10 GΩ. The frequency response of the probe was measured by applying a triangle wave to the case (the eighth pin of the 3523 amplifier), which acted as a small capacitor to the input. The rise time of the square-wave response thus obtained was <200 μs when

compensated by a frequency response correction circuit. The background noise level was ~0.5 pA (peak to peak), when the cutoff frequency (−3 dB) for the low-pass filter was set at 1 kHz. Leakage and capacitative currents were eliminated from the records by subtracting averaged records with no channel openings with the aid of a digital oscilloscope (2090-3C; Nicolet Scientific Corp., Northvale, NJ) in conjunction with a microcomputer. The sampling rate in the analog-digital conversion was 10 kHz. The data were stored in floppy disks and later output to an *X-Y* plotter for purposes of illustration. Single channel records were analyzed with the aids of a TRS 80 model III microcomputer (Radio Shack, a Division of Tandy Corp., Fort Worth, TX).

Using the half-amplitude threshold analysis (Colquhoun and Sigworth, 1983), we determined the transitions between the conducting (open) and nonconducting (shut or blocked) states. In the control, the mean duration of the conducting states will be defined as the lifetime of the open state. In the presence of 9-AA, the single channel made rapid transitions between the open, conducting state and the open, nonconducting state, interspersed with relatively long intervals of no channel activity. These isolated instances of rapid transitions will henceforth be referred to as bursts. These phenomena are depicted in Fig. 1 *B2* and interpreted with a sequential open channel block mechanism (see Scheme 1 in Results). The open, conducting state is an unblocked state and will be henceforth referred to as the open state. The open, nonconducting state is a blocked state and will be referred to as the blocked state. Thus, a burst consists of many rapid transitions between the open and blocked states, and is separated from another burst by a relatively long interval of no channel activity. This relatively long interval, for which there is no channel activity, will be referred to as the shut state.

Since a given burst generally has several open-to-blocked and blocked-to-open transitions, there are several intervals spent in the open state and several intervals spent in the blocked state during a burst. The blocked time (t_b) will be defined as the interval between the open states, and the open time (t_o) will be defined as the interval between the nonconducting states. The shut state and the blocked state are both nonconducting. Therefore, the first open time of a burst is the interval between the shut state before the burst and the first blocked state, the second and subsequent open times are the intervals between the blocked states of the burst, and the final open time of the burst is the interval between the last blocked state of the burst and the shut state after the burst. The time from the start of the first conducting state to the end of the last conducting state was defined as the burst time (t_{burst}).

When the membrane patch contains more than one channel, the determination of the transitions between various states could become ambiguous because of the overlapping events. To reduce the error arising from overlapping events, only patch recordings that had fewer than 7% of overlapping opening events were selected for the control records. The overlapping events that were discarded therefore had a minimal effect on the estimate of the average open time. In the presence of drug, the error in estimating the dwell times in each state, because of multiple channels in the patch, must be minimal for the following three reasons. First, we did not attempt to interpret shut times between bursts, because it was not possible to know for certain whether a burst originated from the same channel opening as the previous burst. Second, no overlapping events were observed, presumably because the number of channel opening events (voltage gated) was reduced by the drug, decreasing the chance of overlap. Finally, the gap within the burst induced by the drug was on the average 3.5 times shorter than the gaps between bursts. We assumed that repetitive opening and closing within a particular burst represents the blocking and unblocking of a single channel, rather than the closing of one channel and the opening of another.

The effect of limited time resolution of a current measurement on the result of a kinetic analysis of the open and blocked times is another important factor to be considered. As pointed out by Neher (1983), the failure of the recording system to detect short events (openings or blockings) has two serious effects on the result of the measurement of the open and blocked times. First, events are lacking in the early bins of histograms (which are usually eliminated from curve fitting). Second, every missed opening leads to an erroneously large value in the measurement of the particular blocked interval during which this opening occurred, and vice versa. Thus, both mean open times and closed times are affected. We did not attempt to correct the errors associated with the limited frequency response in this paper.

Both the curve-fitting method and the average method were used in estimating the t_o and t_b values. For the first method, the mean dwell time was obtained from the fit of the histogram to exponentials for the open times or the blocked times. The open time histogram could be fitted to a single-exponential function with the least-squares method. The time constant for the least-squares exponential fit represented the mean open time (τ_o). Similarly, the mean blocked times (τ_b) were estimated by the same method. There are two sources of error for the curve fitting of the histogram. First, at a tail of the histogram, fitting is least reliable because of the small sample number. Second, the first bin could be underestimated because of the limited frequency response of the recording system, as discussed above. Because of these complications, the fitting was done between the second bin and the bin at which the numbers of events decayed to 10% of the total sample numbers. The second method was to calculate the arithmetic mean of the open times or the blocked times. In general, these two methods gave similar values for the open times or blocked times. When the open or the blocked events were not sufficient in number to construct a histogram or when the mean burst time was estimated, only the average method was used.

The temperature of the chamber was controlled by a Peltier device and measured with a thermocouple probe (BAT-12; Bailey Instruments, Saddlebrook, NJ). The temperature was kept within ±0.5°C of the desired setting, and most experiments were performed between 5.6 and 14°C.

RESULTS

9-AA Caused Flickering in the Single Na Channel

When the excised membrane patch was depolarized to −55 mV or more positive potentials, an inward-going, pulse-like event was observed. This inward-going current had a rather uniform amplitude but varied in the duration at a given membrane potential (Fig. 1*A*). Analogous to other preparations, this pulse-like event has been interpreted as ion flow through an individual channel. The inward currents shown in Fig. 1 were undoubtedly associated with voltage-gated Na channels because they were totally eliminated by an external application of 100 nM tetrodotoxin (not shown here), a toxin that selectively blocked Na channels (Narahashi, 1974). Before the treatment with NBA, the lifetime of the open channel was short, lasting a few milliseconds. After the treatment with NBA, its lifetime became longer, lasting several tens of milliseconds (Fig. 1*A*). Following the introduction of 30 μM 9-AA to the cytoplasmic side of membrane, a drastic change in the current pattern of single channel openings took place: the single channel current no longer appeared as a simple square wave, but showed a bursting or flickering behavior (Fig. 1*B*). The bursting behavior is

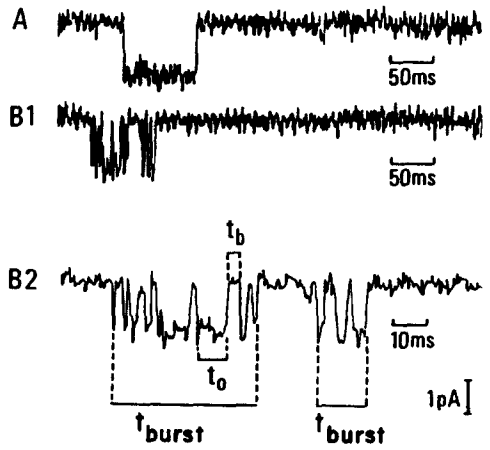

FIGURE 1. Examples of single Na channel currents recorded before (*A*) and during (*B*) perfusion with 30 μM 9-AA. The same record as *B1* is shown in *B2* with expanded time scales. Both *A* and *B* were obtained from a single inside-out membrane patch pretreated with 300 μM NBA. The Na channel currents were obtained by depolarizing the membrane to −40 mV from a holding potential of −100 mV. Temperature, 6°C. In *B2*, the time intervals corresponding to the variables t_o (open time), t_b (block time), and t_{burst} (the duration of a burst) are indicated.

interpreted as a manifestation of the blocking and unblocking of a single open channel by 9-AA molecules. The long duration of opening can greatly facilitate the analysis of discrete blocking and unblocking events when the channel is exposed to a channel blocker. Therefore, all results in this paper were obtained from the NBA-treated membrane patches.

As can be seen in expanded traces (Fig. 1 *B2*), there are two bursts, separated by a relatively long interval with no channel activity (the gap between two bursts). The gap between bursts was, on the average, three to four times as long as the gap (the blocked duration) within unambiguous bursts. Thus, the burst could be clearly identified.

Within a burst, individual short open pulses have the same amplitude as the control current, whereas the intervening blocked states seem to have zero conductance (when the limited frequency response of the recording system was taken into account). At −40 mV, the mean amplitude of the control single channel current was 1.70 pA, and that during the conducting state in the presence of 9-AA was 1.62 pA (temperature at 6°C). Thus, the single channel conducts normally when it is in the unblocked state, whereas it loses its conductance totally when in the blocked state.

Kinetic Analysis of 9-AA Block

When the gaps within the burst are short and there are no overlapping events within the burst, one can safely conclude that all of the openings within one burst originate from one channel, even if there are more than one channel present in the patch (Colquhoun and Sigworth, 1983). Both conditions are met, as shown in Fig. 1 *B2*. Thus, all of the openings within bursts are considered to

originate from the same single channel. The short openings and closings within a burst reflect the unblocking and blocking of a gate-open channel caused by 9-AA molecules, as in the case of a simple ion channel-blocking mechanism.

On the basis of studies of 9-AA block of macroscopic Na currents in squid axons, Cahalan (1978) and Yeh (1979) concluded that 9-AA blocks open Na channels. The following version of a sequential model for open channel block was simplified by lumping all closed states of the Na channel into one shut state.

$$\text{shut} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open} \underset{l}{\overset{k[B]}{\rightleftharpoons}} \text{blocked} \qquad \text{(Scheme 1)}$$

Here, α is the apparent opening rate constant, β represents the rate constant governing the transition from open to the last shut state prior to the open state of the Na channel, $[B]$ is the concentration of blocker molecule, and k and l are the blocking and unblocking rate constants. This model served as the basis for the kinetic analysis of 9-AA block of the single Na channel.

The purpose of this section is to demonstrate that the two rate constants governing the blocking reaction can be accurately estimated from the single channel recordings. Given that bursting occurs, this can be done simply by measuring the duration of the open and the blocked states within bursts. Therefore, this analysis depends on whether one can clearly identify a burst. For 9-AA, we found that this is the case for the following reasons. According to Scheme 1, the transitions between the blocked state and the shut state are not permissible. However, one can identify these two states (even though both the blocked state and the shut state do not carry current) if they have very different mean durations, because the histograms of the mean durations of all nonconducting states (the shut and the blocked states) will be distributed as the sum of two exponentials, the time constants of which are equivalent to the mean durations of the shut and the blocked states. We found this was indeed the case. The time constants describing each exponential component are estimated to be 1 and 3–4 ms. Since they differed by a factor of 3.5, we have developed a criterion for deciding whether a nonconducting state belongs within or between bursts. The criterion is that a gap (the duration of any nonconducting state) that is 3.5 times greater than the mean blocked duration in unambiguous bursts will be treated as the gap between bursts. With this method, the probability that a blocked state (gap within bursts) is mistaken for a shut state (gap between bursts) is calculated to be 3%.

According to Scheme 1, the duration of the channel in the open state (t_o) is randomly distributed with a mean duration of the reciprocal of the sum of rates leaving that state. In other words, the reciprocal of the mean open time (τ_o) follows Eq. 1:

$$1/\tau_o = \beta + k[B]. \qquad (1)$$

The duration of the channel in the blocked state (t_b) is randomly distributed with the mean duration of the reciprocal of the rate leaving that state. In other words,

the reciprocal of the mean blocked time (τ_b) follows Eq. 2:

$$1/\tau_b = l. \tag{2}$$

By measuring the open time of the short pulse (t_{open} or t_o) and the blocked time ($t_{blocked}$ or t_b) (Fig. 1*B2*), one can estimate the individual rate constants governing drug/channel interaction.

Fig. 2 shows the histograms for the open times and the blocked times. These histograms were constructed from data obtained with an NBA-pretreated single patch during perfusion of 60 μM 9-AA. The histograms for the open times and for the blocked times followed an exponential distribution. The exponential least-squares fit of data gave the mean open time and the mean blocked time as 1.4 and 1.0 ms, respectively. A direct calculation using the average method gave the mean open time as 1.6 ms and the mean blocked time as 1.2 ms.

CONCENTRATION DEPENDENCE In our sequential model, we have assumed that one drug molecule binds to one Na channel. According to Eqs. 1 and 2, then, the reciprocal of the open time is a linearly concentration-dependent variable, but the reciprocal of the mean blocked time is not concentration dependent at all. The data in Fig. 3*A* demonstrated that the blocked time had virtually no significant concentration dependence. This is in accord with the proposed model. The slight variation in τ_b^{-1} over concentration ranges from 30 to 100 μM was within the experimental error. The data in Fig. 3*B* showed that the τ_o^{-1} values increased linearly with increasing the blocker concentration. The

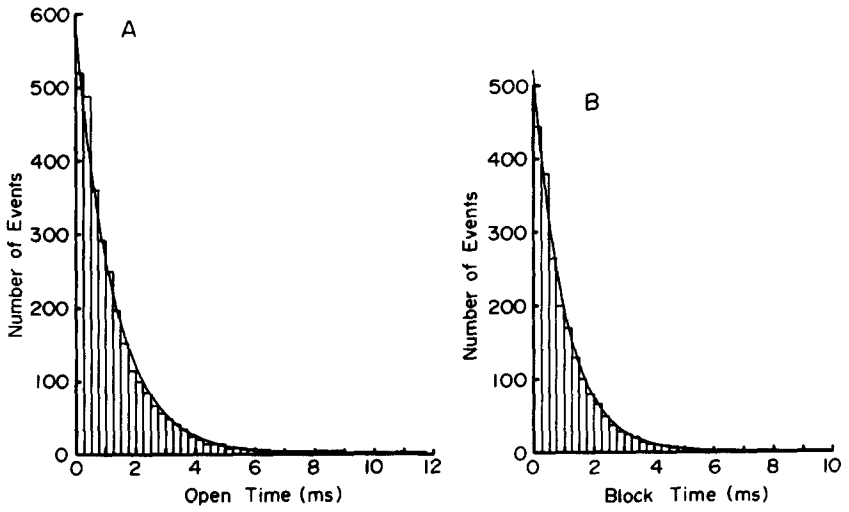

FIGURE 2. Histograms of time intervals for t_o (*A*) and t_b (*B*) (for definitions of t_o and t_b, see Fig. 1) in the presence of 60 μM 9-AA. The single channel currents were activated by depolarizing the membrane to −40 mV from a holding potential of −100 mV. The membrane patch was pretreated with 300 μM NBA. The number (*N*) of events having a duration longer than that indicated on the abscissa is plotted against the duration (*t*). The curves were drawn by the equation $N = 600 \exp(-t/1.4)$ for the open time (*A*) and $N = 578 \exp(-t/1.0)$ for the blocked time (*B*). Temperature, 16°C.

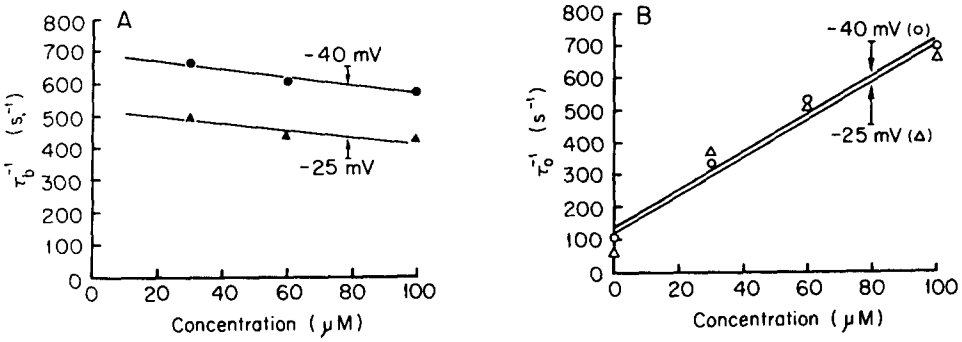

FIGURE 3. Concentration dependence of the reciprocal of the mean blocked time, τ_b^{-1} (*A*) and the reciprocal of the mean open time, τ_o^{-1} (*B*) at −25 and at −40 mV. Lines are the linear regression with the equation $\tau_o^{-1} = k[B] + \beta$, where $[B]$ is the concentration of 9-AA (μM), β is the rate constant (s^{-1}) at 0 μM concentration of 9-AA, and k is the slope. The values are: $\beta = 135\ s^{-1}$, $k = 5.9 \times 10^6\ M^{-1}s^{-1}$ (○); $\beta = 123\ s^{-1}$, $k = 5.8 \times 10^6\ M^{-1}s^{-1}$ (△). The reciprocal of the mean blocked time has very little concentration dependence. Temperature, 6°C.

forward rate constant, k, and the channel closing rate constant, β, were obtained from Eq. 1 because the slope of the line relating τ_o^{-1} to the drug concentration, $[B]$, corresponds to k and its intercept at the ordinate corresponds to β. The values for k were $5.8 \times 10^6\ M^{-1}s^{-1}$ at −25 mV and $5.9 \times 10^6\ M^{-1}s^{-1}$ at −40 mV, and values for β were $123\ s^{-1}$ at −25 mV and $135\ s^{-1}$ at −40 mV.

VOLTAGE DEPENDENCE The voltage dependence of the blocking reaction was also examined. Fig. 4, *A–C*, illustrates traces of single channel currents in the presence of 60 μM 9-AA at different membrane potentials. One can see that the blocked time markedly decreased as the membrane was hyperpolarized, while the open time was only slightly increased. This visual impression of the voltage dependence of the blocked times and open times was verified by further kinetic analysis.

Fig. 5 summarizes the voltage dependence of the reciprocal mean open times

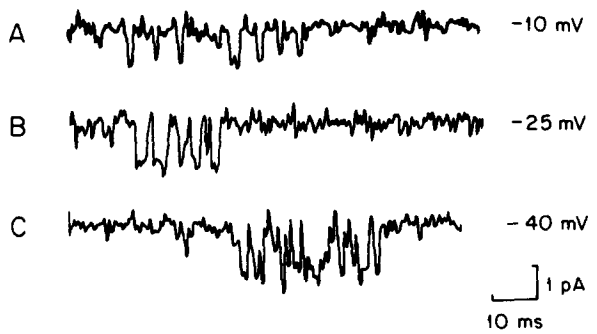

FIGURE 4. Voltage dependence of channel flickering. Representative traces at −10 (*A*), −25 (*B*), and −40 mV (*C*) in the presence of 60 μM 9-AA. Note that the blocked time increases as the membrane is depolarized, while the open time is not appreciably different. Temperature, 6°C.

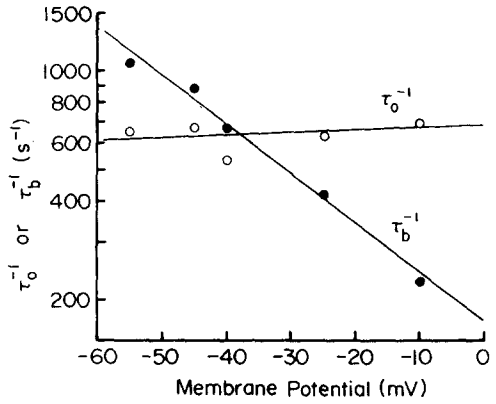

FIGURE 5. Voltage dependence of the τ_o^{-1} (○) and τ_b^{-1} (●). Data points were fitted by the equation τ_o^{-1} or $\tau_b^{-1} = A \cdot \exp(a \cdot E)$, where E is the membrane potential (mV), A is the rate constants at 0 mV, and a is a slope factor. The following values were used in this example: A = 166 s^{-1}, a = −0.035 mV^{-1} for τ_b^{-1}; A = 673 s^{-1}, a = 0.0019 mV^{-1} for τ_o^{-1}. Data were obtained from a single inside-out membrane patch during perfusion of 60 μM 9-AA. Temperature, 6°C.

and the reciprocal mean blocked times. The reciprocal mean blocked time, $1/\tau_b$, decreased as the membrane was depolarized, and this voltage dependence is expressed by:

$$\tau_b^{-1} = A \exp(aE), \tag{3}$$

where E is the membrane potential in millivolts, A is τ_b^{-1} at 0 mV, and a is the slope factor. In the illustrated case, A was 166 s^{-1} and a was −0.035 mV^{-1}. The reciprocal mean open time was only weakly voltage dependent, and the voltage dependence was fitted by an equation similar to Eq. 3. In the example shown in Fig. 5, the reciprocal mean open time at 0 mV was 673 s^{-1} and the slope factor was 0.0019 mV^{-1}.

COMPARISON OF 9-AA-INDUCED BURST DURATION WITH NORMAL CHANNEL LIFETIME Another prediction from the sequential model is that the mean burst time in the presence of blocker should be longer than the mean lifetime in the

TABLE I

Comparison of the 9-AA (30 μM) -induced Burst Time with Normal Channel Lifetime

Life or burst time			Ratio			
Before	During	After	During/ Before	After/ before	E_m	Tempera- ture
	ms				*mV*	°C
6.7	11.3	7.8	1.69	1.16	−40	14
16.9	24.2	15.4	1.43	0.91	−25	6
21.1	50.2	27.6	2.38	1.31	−25	9
		Mean	1.83	1.13		

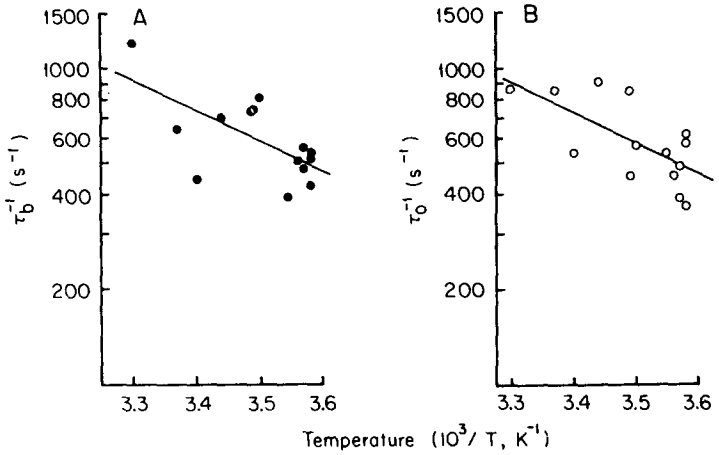

FIGURE 6. Temperature dependence of τ_b^{-1} (*A*) and τ_o^{-1} (*B*) measured in the presence of 30 μM and at −25 mV. The temperature dependence was estimated using a linear regression and the slope of the line fitted to the compiled data from nine different inside-out membrane patches was −2,250°K for both τ_b^{-1} and τ_o^{-1}, which corresponds to a Q_{10} of 1.31.

absence of the drug, because the termination of a burst is not simply by leaving a single open state, but rather leaving a set of states, the open and the blocked states. Since the channel closing rate constant, β, was considerably less than either the blocking rate, $k[B]$, or the unblocking rate, l, the reciprocal mean burst duration could be approximated as follows:

$$\tau_{burst} = \beta^{-1}(1 + k[B]/l). \quad (4)$$

The experimental results were in agreement with this prediction (Table I). Values of the mean lifetime and the mean burst time, measured with three NBA-treated membrane patches, are shown in Table I. Even though the mean lifetime varied from one patch to the other, the mean burst time in the presence of 9-AA was significantly longer than the mean lifetime of the open channel in the absence of drug. On the average, the burst time was 80% longer than the normal open channel lifetime. This effect of 9-AA was fully reversible.

TEMPERATURE DEPENDENCE Fig. 6 shows the temperature dependence of the reciprocal of the mean block time, τ_b^{-1}, and the reciprocal of the mean open time, τ_o^{-1}, in the presence of 30 μM 9-AA. Linear regression analysis gave estimates of the Q_{10} for τ_b^{-1} and τ_o^{-1} of 1.31. The low Q_{10} for τ_b^{-1} suggests that the unblocking rate constant, l, is diffusion controlled. Regarding the Q_{10} for τ_o^{-1}, the interpretation is complicated by the fact that τ_o^{-1} is a function of two rate constants, i.e., the channel closing rate, β, and the blocking rate constant, k. Each rate constant could have a different temperature dependence.

DISCUSSION

Several features of the 9-AA block of Na channels were revealed in this study. First, 9-AA caused the channel to flicker, i.e., the channel underwent repetitive rapid transitions between fully conducting (open) and nonconducting (blocked)

states. The rapid transitions produced a bursting behavior during a single channel opening. Second, the mean open time within the burst became shorter as the drug concentration was increased, whereas the mean blocked time was concentration independent. Third, the unblocking rate constant was potential dependent, and it was increased with hyperpolarization, while the blocking rate constant was only weakly potential dependent. Fourth, the mean burst time in the presence of drug was longer than the mean lifetime of an open channel in the absence of drug. All of the above features of 9-AA block of single Na channels are compatible with a sequential model in which 9-AA molecules bind to and block the open channels. In addition, this model requires that the blocked channel not close until it has freed the drug molecule from the blocking site in the channel.

The observation that the mean open time of the blocked channel has little voltage dependence merits some comment. According to Eq. 2, the open time is given by the sum, $k[B] + \beta$, where $k[B]$ is the product of the blocking rate constant k and the drug concentration $[B]$, and β reflects the rate of returning to a state prior to the opening of the channel (which has been referred to as deactivation). There are two ways in which this lack of voltage dependence of the open time can arise: (*a*) neither $k[B]$ nor β varies with the potential; (*b*) $k[B]$ and β both vary with the potential in such a way that their sum remains constant. Mechanism *b* implies that k and β should have an opposing voltage dependence. In fact, our evidence suggests that k is potential independent. The value of k is calculated from the slope of the τ_o^{-1} vs. concentration relation (Fig. 3). This slope was identical at −40 and −25 mV. In addition, β is only weakly potential dependent. The intercept gives estimates of β that are 135 and 125 s^{-1} at −40 and −25 mV, respectively. This leads us to believe that mechanism *a* is applicable at least between −25 and −40 mV.

Outside this potential range, a direct measurement of k and β has not been made. With regard to β, Horn et al. (1984) suggested that β, in the GH_3 cell, had a bell-shaped voltage dependence between −60 and −10 mV. However, if one inspects their data more closely, one finds that the deactivation rate constant, β, is only weakly voltage dependent. If such a voltage dependence is applicable to the neuroblastoma cell, then the lack of variation of τ_o^{-1} would imply a weak potential dependence for k. In their experiments, Na inactivation was also removed by NBA treatment.

In agreement with the present findings on single channel currents, Cahalan (1978) and Yeh (1979, and unpublished data), studying macroscopic Na currents in pronase-treated squid axons, showed that the forward rate constants describing 9-AA block are potential independent. This agreement is impressive considering the differences in preparation and methodology. Also, the range of potentials in which block was measured varied between 0 and +100 mV for the macroscopic currents and between −55 and −10 mV for single channel currents. In addition, both macroscopic and single channel studies have shown that the unblocking rate constant is highly potential dependent. This potential dependence of unblocking rate constants could account for the potential dependence of an equilibrium block in the presence of 9-AA (Yeh, 1979).

The voltage-dependent block of ionic channels by various ions and drugs has

often been modeled with a two-barrier, one-site model (Woodhull, 1973). According to this model, for a blocker to block a channel, the blocker has to hop over the first barrier in order to bind its blocking site (energy well); for an unblocking action to occur, the drug molecule in the blocking site is assumed to hop over the first barrier back to the original side of the membrane, rather than over the second barrier, this barrier being an unsurmountable one. In addition, the first barrier is, in general, assumed to be a symmetrical one, i.e., the electrical distance from the membrane surface to the peak of the energy barrier equals the distance between the peak of the energy barrier and energy well (the blocking site). QX-222 represents an example of such blockers. From studies on the QX-222 block of the ACh-activated single channels in frog endplates, Neher and Steinbach (1978) found that blocking and unblocking rate constants have essentially equal but opposite dependences. This was interpreted by using a model in which a QX-222 molecule has to cross a symmetrical barrier to reach the binding site.

9-AA block of Na channels differs from the classic model for ionic block in that 9-AA molecule has to hop over an asymmetrical barrier to bind to the blocking site. The voltage dependence of the blocking rate constant is expressed by:

$$k(E) = k(0) \exp(\delta_1 EF/RT), \tag{5}$$

where $k(0)$ is the blocking rate constant at 0 mV, and $k(E)$ is the blocking rate constant at any membrane potential, E. The electrical distance, δ_1, is measured from the cytoplasmic surface to the peak of the first barrier; F, R, and T have their usual meanings. The average value of $k(0)$ was $1.0 \times 10^7 \pm 0.3$ $M^{-1}s^{-1}$ (mean ± SD; $n = 4$). The average value of δ_1 was 0.05 ± 0.10 (mean ± SD; $n = 4$). The result suggests that the peak of the first barrier is very close to the cytoplasmic membrane surface.

In contrast to the blocking rate constant, the unblocking rate constant is highly potential dependent, and its voltage dependence is expressed by:

$$l(E) = l(0) \exp(-\delta_2 EF/RT), \tag{6}$$

where $l(E)$ and $l(0)$ are the unblocking rate constants at any membrane potential, E and 0 mV, respectively. The average value of $l(0)$ from four different membrane patches was 214 ± 30 s^{-1} (mean ± SD). The electrical distance, δ_2, is measured from the peak of the first barrier to the energy well (binding site), being 0.63 ± 0.11 (mean ± SD). The result indicates that the unblocking rate constant is contributing most of the voltage dependence of the equilibrium dissociation constant, K_d. The K_d value is defined as l/k, and the electrical distance for the blocking site, δ, is the sum of $\delta_1 + \delta_2$. The K_d values are estimated to be 21 and 65 μM at 0 and −40 mV, respectively. The δ value is 0.68, which suggests that 9-AA binds to a first-order blocking site located 68% of the way through the membrane field from the cytoplasmic surface.

This difference in the voltage sensitivity of each rate constant has been interpreted to represent a difference in the energy profiles of these ionic channels. This difference in the energy profiles may, in turn, reflect an intrinsic

difference in the architecture of ionic channels, or may also reflect a dynamic nature of channel properties (Läuger, 1980). Other factors, such as conformational changes of the blockers and diffusion, could also play an important role in determining the voltage dependence of channel blocking action. In the case of the block of sarcoplasmic reticulum K channels by *bis*-quaternary ammonium ions, the blocking rate constant had prominent voltage dependence, whereas the unblocking rate constant lacked any voltage dependence (Miller, 1982). To account for the voltage dependence of blocking rate, Miller (1982) put forward a hypothesis that the rate-limiting step for blocking action is the conformational change of the blocker molecule in response to voltage change.

Several lines of evidence suggest the importance of the diffusion process in controlling the drug blocking action. In many types of ionic channels, the blocking rate constant does not seem to depend on the structure of blockers. In this study, the forward blocking rate constant for 9-AA binding has been estimated to be on the order of 1×10^7 $M^{-1}s^{-1}$. This value is within the same order of magnitude as that for QX-222, QX-314 (Neher and Steinbach, 1978), barbiturates, procaine (Adams, 1976, 1977), and octylguanidine (Farley et al., 1981) in blocking ACh-activated channels from the outside, and that for saxitoxin, tetrodotoxin (Wagner and Ulbricht, 1975), and pancuronium (Yeh and Narahashi, 1977) in blocking Na channels, and that for TEA in blocking K channels in nerve membranes from the inside (Armstrong, 1966). The similar rate of block by such a large class of different substances suggests that the binding reaction is probably diffusion controlled. This notion is also supported by the observed low temperature dependence for the blocking and unblocking rate constant, with a Q_{10} of ~1.3 for both rate constants. This low temperature dependence is characteristic of a free diffusion process (Robinson and Stokes, 1970).

The burst period in the presence of 9-AA is prolonged by a factor of the $k[B]/l$, as expected from the sequential model (Eq. 4). Since the blocking rate constant, k, is voltage independent and since the unblocking rate constant, l, is voltage dependent, increasing with hyperpolarization (according to Eq. 6), the factor $k[B]/l$ decreases with hyperpolarization. In other words, the prolongation of the burst period is smaller when the membrane is hyperpolarized than when the membrane is depolarized. This actually is the case, as shown in Table I. 9-AA at 30 μM prolongs the burst period by 56 and 78% at −40 and −25 mV, respectively.

The present results (flickering behavior of currents in the presence of 9-AA, prolongation of open channel lifetime by 9-AA, the concentration dependence of the blocking rate, and the lack of concentration dependence in the unblocking rate constant) are all compatible with the sequential model for 9-AA block of Na channels. How the inactivation gate modulates the drug/Na channel interaction at the single channel level will be the subject of future studies.

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