Dynamics of 9-Aminoacridine Block of Sodium Channels in Squid Axons

JAY Z. YEH
From the Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611

ABSTRACT The interactions of 9-aminoacridine with ionic channels were studied in internally perfused squid axons. The kinetics of block of Na channels with 9-aminoacridine varies depending on the voltage-clamp pulses and the state of gating machinery of Na channels. In an axon with intact h gate, the block exhibits frequency- and voltage-dependent characteristics. However, in the pronase-perfused axon, the frequency-dependent block disappears, whereas the voltage-dependent block remains unchanged. A time-dependent decrease in Na currents indicative of direct block of Na channel by drug molecule follows a single exponential function with a time constant of 2.0 ± 0.18 and 1.0 ± 0.19 ms (at 10°C and 80 mV) for 50 and 100 μM 9-aminoacridine, respectively. A steady-state block can be achieved during a single 8-ms depolarizing pulse when the h gate has been removed. The block in the h-gate intact axon can be achieved only with multiple conditioning pulses. The voltage-dependent block suggests that 9-aminoacridine binds to a site located halfway across the membrane with a dissociation constant of 62 μM at 0 mV. 9-Aminoacridine also blocks K channels, and the block is time- and voltage-dependent.

Local anesthetics block the excitability of nerve by blocking Na channels (Taylor, 1959). The block may exhibit both frequency- and voltage-dependent features. The frequency-dependent block arises from the interaction of binding reaction with channel gating (Courtney, 1975), and the voltage-dependent block is ascribed to the binding reaction which is itself voltage-dependent (Strichartz, 1973). The receptor for the local anesthetic molecule is thought to be located inside the Na channel (Strichartz, 1973; Hille, 1977). The cationic local anesthetic molecule is thought to gain access to the receptor via the inner mouth of the channel only when the channel is open. Thus, the drug-receptor binding and the drug effect on the gating mechanism are two factors that are important in modulating the voltage- and frequency-dependent block.

This paper presents a detailed analysis of the frequency-dependent block of Na channels using 9-aminoacridine (9-AA) as an example. Though the frequency-dependent block of Na channels has been studied in myelinated nerve preparations (Strichartz, 1973; Khodorov et al., 1976; Courtney, 1975; Hille, 1977), squid axon preparation offers two distinct advantages for these studies: First, internal perfusion provided a direct delivery of drug to the inside of axon membrane; and second, the h gate may be removed by pronase treatment.
(Armstrong et al., 1973), which gives another dimension for kinetic analysis of drug-channel interaction without complication from Na inactivation. Pronase treatment removes the frequency-dependent block of Na channels by 9-AA but does not affect the voltage-dependent block. The results with 9-AA are best explained by a model with a single binding site halfway down the Na channel. The h gate can modulate the frequency-dependent block by controlling the accessibility of channels to a drug molecule and by trapping the molecule in the channel as the h gate closes. The m gate also affects the frequency-dependent block in a similar fashion. A short report of part of this investigation has appeared elsewhere (Yeh and Narahashi, 1975, 1976).

**METHODS**

Experiments were performed with giant axons isolated from the squid *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, Mass. Both intact axons and axons internally perfused by the roller technique of Baker et al. (1961) were used. The axial wire voltage clamp used in the present study was described previously (Wu and Narahashi, 1973). The response time of the clamp was approximately 10 µs (10-90% of step-pulse command). About two-thirds of the measured series resistance of 3-4 Ω cm² was compensated by a feedback circuit in all experiments.

The axons were perfused externally with artificial seawater (ASW) with the following ionic concentrations (mM): Na⁺, 450; K⁺, 10; Ca²⁺, 50; Cl⁻, 576; Hepes buffer, 5. In several experiments an external bathing medium with a low sodium concentration (50 mM) was made by equimolar replacement of 400 mM sodium chloride with tetramethyl-ammonium chloride. External pH was adjusted to 8.0 in all cases. The standard internal solution had the following composition (mM): Na⁺, 50; K⁺, 350; glutamate, 320; F⁻, 50; sucrose, 333; phosphate buffer, 15; and was adjusted to a pH of 7.3. To eliminate potassium current, tetraethylammonium (TEA) was added to the standard internal solution at a concentration of 20 mM. To eliminate sodium current, 300 nM tetrodotoxin (TTX) was added to ASW. All experiments were performed at a constant temperature of 9-11°C.

9-AA hydrochloride was purchased from K and K Laboratories Inc. (Plainview, N. Y.). 9-AA with pKa of 9.99 is almost entirely ionized as a cationic form at the pH of 7.3. The partition coefficient of 9-AA, measured between de-acidified olive oil and water at pH 7 and 20°C, is 1.2 (Albert, 1966).

**Data Analysis, Terminology, and Computations**

Oscilloscope records of membrane currents and potentials were photographed on 35-mm film and analyzed manually with the aid of a Hewlett-Packard digitizer and a Hewlett-Packard 9821 programmable calculator with a 9864 digital plotter output (Hewlett-Packard Co., Palo Alto, Calif.).

The term “channel” as adopted in this paper is operationally defined as the pathway in axon membranes through which ions pass. “Gate” or “gating” refers to the normal processes by which ionic channels allow ions to pass across the membrane.

A two-barrier model for ionic block of sodium channels (Woodhull, 1973) was adopted using the following equation:

\[
\frac{I_{Na}(\text{in 9-AA})}{I_{Na}(\text{control})} = \frac{1}{(1 + [x]K_a \exp(\delta E_m F/RT))}
\]

where \(x\) is the internally applied drug concentration, \(K_a\) is the binding constant at 0 mV, \(E_m\) is the membrane potential, \(\delta\) is the fraction of the membrane potential which affects
Y~H 9-Aminoacridine Block of Na Channel~

the binding reaction, a dimensionless parameter with value between zero and unity measuring from inside, and $R$, $F$, and $T$ have their usual meanings.

**RESULTS**

9-AA affected both the sodium and potassium conducting systems of squid axon membranes when applied either internally or externally. 9-AA inhibited the sodium channel in a manner dependent on the frequency and amplitude of pulses. This frequency-dependent (or use-dependent) block of the sodium channel disappeared after the sodium inactivation mechanism was removed by pronase treatment. 9-AA also blocked the potassium channel and a detailed analysis is not reported here. Most studies of the kinetics of block were done with internal perfusion of 9-AA.

**9-AA Block of the Sodium Channel Depends on the Previous History**

The decrease in the sodium current by 9-AA depended on the previous history of the axon. Fig. 1 shows the dynamics of block of sodium currents by $3 \times 10^{-5}$ M 9-AA. The initial decrease in the sodium current was very small when the axon had been held at $-80$ mV for several minutes without being subject to conditioning depolarization even though the axoplasmic concentration of 9-AA was presumably at equilibrium. This is called the *initial block*. The degree of block was increased by applying conditioning depolarizing pulses and reached the steady-state level after about 20 pulses at a frequency of 1 Hz. This is

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Enhancement of 9-AA block by repetitive conditioning potentials. The upper part of this figure shows the voltage-clamp wave forms composed of 8-ms conditioning depolarizations to 80 mV at a frequency of 1 Hz followed by an 8-ms test pulse to $-20$ mV. Holding potential $-80$ mV. The inward Na currents associated with the test pulse before and during internal perfusion of $3 \times 10^{-5}$ M 9-AA are shown in the lower part. The number of conditioning pulses preceding the test pulse is labeled beside the current traces.
conditioned block. The steady-state level of conditioned block depended upon the voltage of the conditioning pulses, as will be discussed in detail later.

If the membrane potential was held for several minutes at a slightly hyperpolarized level (−70 to −80 mV) after the conditioned block, the sodium current was partly restored. This is called the resting block. The degree of the resting block was always greater than the initial block, as shown in Table I. Fig. 2 illustrates the comparison between the resting block and conditioned block. The reversal potential for sodium currents estimated from the family of sodium current was not appreciably affected by internal application of 3 × 10⁻⁵ M 9-AA during rest or after having been conditioned at 80 mV. Therefore, the rest and conditioned blocks are the result of a decrease in sodium conductance.

The Onset of the Conditioned Block

FREQUENCY AND CONCENTRATION DEPENDENCE The time-course of block of the sodium conductance by repetitive pulsing to 80 mV is shown in Fig. 3. The conductance decreased exponentially with time when pulse frequency was constant and the time constants were shortened by increasing the concentration of 9-AA and by an increase in the frequency of pulsing. Curves I and II of Fig. 3 are drawn according to the formula \[ A - (A-1) \exp \left( -t/\tau_1 \right) \] where \( \tau_1 \) is the time constant for the onset of block, \( t \) is the time, and \( A \) is the steady-state block of sodium current as a fraction of the initial amplitude. The time constants at +80 mV being 5.71 ± 0.24 and 1.87 ± 0.09 s (n = 3), respectively at 3 × 10⁻⁵ M and 1 × 10⁻⁴ M, when pulse frequency is at 1 Hz. An increase in the frequency of pulsing shortened the time constant of block at +80 mV from 5.71 ± 0.24 s (n = 4) at a frequency of 1 Hz to 3.10 ± 0.18 s (n = 4) at 2 Hz (not shown in this figure). The recovery from the conditioned block, on the contrary, was rather independent of the concentration of 9-AA as illustrated by curves III and IV. Curves III and IV are drawn according to the formula \[ 1 - (1-B) \exp \left( -t/\tau_2 \right) \] where \( \tau_2 \) is the time constant for the recovery of sodium current from the conditioned block B. The time constant was estimated to be 4.85 ± 2.50 (n = 4) and 3.95 ± 0.65 s (n = 4) for 3 × 10⁻⁵ M and 1 × 10⁻⁴ M 9-AA, respectively. This phenomenon will be discussed later.

PREPULSE DEPENDENCE The onset of frequency-dependent block is also dependent upon the amplitude of prepulse potentials. The pulse sequence used to test prepulse dependence is shown in Fig. 4 A. After various numbers of
FIGURE 2. Effect of conditioning pulses on families of sodium currents before (A) and during (B and C) internal perfusion with 3 x 10^-5 M 9-AA. The axon is held at a holding potential of -80 mV and depolarized by test pulses (E_t) ranging in 20-mV steps from -60 to +80 mV. In B, the test pulse from the holding potential is applied at intervals of 2 min and in C the test pulse is preceded by 20 conditioning 8-ms pulses to +80 mV at a frequency of 1 Hz. The voltage-clamp wave forms are indicated in the lower section.

FIGURE 3. Onset of 9-AA block by pulsing to +80 mV and relief of the conditioned block by pulsing to -20 mV. Both pulses were applied at an interval of 1 s. The voltage-clamp wave forms are shown on the upper part, and outward Na currents associated with step depolarizations to +80 mV are progressively decreased and inward currents associated with -20 mV are gradually increased by repetitive pulsing as shown in the inset. For each of 3 x 10^-5 M and 1 x 10^-4 M 9-AA, the peak Na current associated with each pulse is normalized to the value obtained after a long rest in the presence of respective concentrations of 9-AA. Curves are drawn according to the formula described in the text.
prepulse/conditioned pulse pairs, a standard test pulse is given to measure the peak sodium current. Between each trial a 2-min rest is allowed to avoid the cumulative effect. The normalized peak Na current is plotted against the number of pulse pairs as shown in Fig. 4 B. The onset of block was faster and the steady-state block was greater for prepulsing to more hyperpolarizing potentials. The normalized onset of conditioned block is calculated and plotted as a function of prepulse potentials as shown in Fig. 4 C. The prepulse dependence for onset of conditioned block is very similar to that for normal Na inactivation ($h_\infty$) as illustrated in the same figure. At $E_{pp}$ more negative than $-40$ mV, the steady-state level of block seemed to be more or less proportional to the steady-state value of normal Na inactivation as well (not shown in Fig. 4 C).

![Diagram](image.png)
Pulse Duration vs. Total Number of Pulses

When a single 30-ms conditioning pulse of 80 mV was compared to five repetitive conditioning pulses, 6 ms to 80 mV (at 1 Hz), the latter pulse schedule was found to be more effective in enhancing the block. Fig. 5 depicts clearly the difference in the effectiveness of these two pulsing schedules. The inward current associated with the test pulse to -20 mV was smaller after multiple short pulses than a single long pulse. This result, together with the result shown in Fig. 4 C, suggests that h gate must be open for drug molecules to block Na channels.

The Steady-State Level of the Conditioned Block Depends on the Voltage

The voltage dependence of conditioned block was evaluated by conditioning the voltage-clamped axon to various step potentials, each 8 ms in duration and at a frequency of 1 Hz. With this pulsing schedule, the steady-state level could be achieved with 20 pulses and is normalized with the inward current associated with the test pulse in the absence of 9-AA. Fig. 6 A shows that this ratio decreases with more positive conditioned potentials ($E_c$). The data are fit reasonably well by Eq. 1. The parameters used to fit curves I and II are as follows: $K_a = 1.2 \times 10^4 \text{ M}^{-1}$ and $\delta = 0.5$; and with $[x]$ equals $3 \times 10^{-5} \text{ M}$ and $1 \times 10^{-4} \text{ M}$ for curve I and II, respectively. However, there are consistent deviations of the data points from the calculated curves at the conditioning potentials more negative than -40 mV, probably because the channels are not fully open in these potential ranges and gating mechanisms of channels are very sensitive to voltage change.

After pronase treatment, the multiple pulses experiment failed to demonstrate the voltage-dependent block (see curve III in Fig. 6 B) because drug molecules come off quickly between pulses. To estimate equilibrium block by 9-AA at various potentials, Na currents were measured at the end of an 8-ms pulse in the absence and presence of 9-AA, and the ratios were plotted as a
function of membrane potential as shown in Fig. 6 B (open squares). It is clear that voltage dependence of 9-AA block remains intact after pronase treatment. The parameters used to yield curve IV are $\delta = 0.5$, $K_a = 1.7 \times 10^4 \text{ M}^{-1}$, and $x = 3 \times 10^{-5} \text{ M}$.

**Figure 6.** Effect of the concentration of 9-AA and the internal perfusion of pronase on the voltage dependence of 9-AA block of Na currents. In the presence of 9-AA, the steady-state block of Na currents was obtained by 20 conditioning pulses to various potentials ($E_c$) at 1 Hz and measured with test pulse to -20 mV. The peak Na currents associated with test pulse were then normalized to the control (in the absence of drug), and the normalized values were plotted as a function of $E_c$ (● and ○ in A and ■ in B). After pronase treatment, Na currents were measured at the end of an 8-ms pulse to various membrane potentials ($E_m$), and the ones in the presence of 9-AA were normalized to the control. The normalized values are illustrated as the open square (○) in B. Curves I, II, and IV are drawn according to Eq. 1 as described in the text. The parameters used to fit the curves were as follows: $K_a = 1.2 \times 10^4 \text{ M}^{-1}$ for curves I and II; $1.7 \times 10^4 \text{ M}^{-1}$ for curve IV; and $\delta = 0.5$ for all cases. Curve III for the solid square (■) is drawn by eye.

**Recovery from the Frequency-Dependent Block**

The degree of recovery from the frequency-dependent block was measured by two methods. The channels were first conditioned to the most blocked state by
pulsing 20 times to +80 mV at 1 Hz. In the resting method, recovery from this conditioned block was evaluated by a constant test pulse to −20 mV at a very low frequency, one every 10 s. In the pulsing method, the degree of recovery from conditioned block was monitored by a constant test pulse to −20 mV after pulsing to various step depolarizations 15 times at 1 Hz. The amplitude of the sodium current associated with the test pulse is a measure of the degree of recovery from the conditioned block.

Fig. 7 A shows that the recovery from the frequency-dependent inhibition is faster in axons with frequent pulsing to −20 mV than in resting axons. The axon pulsed to −20 mV at 1 Hz recovered from the frequency-dependent block caused by $3 \times 10^{-5} \text{ M}\, 9$-AA with a time constant of 4.6 s, whereas the axon at rest recovered with a time constant of 18 s. The time constants for recovery were not appreciably affected by the concentration of 9-AA. For instance, the time constants for pulsing recovery are $4.85 \pm 2.50$ and $3.95 \pm 0.66 \text{ s (n = 4)}$ for $3 \times 10^{-5}$ and $1 \times 10^{-4} \text{ M}\, 9$-AA, respectively. The time constants for rest recovery are $18.5 \pm 2.5$ and $20.0 \pm 3.0 \text{ s (n = 3)}$ for $3 \times 10^{-5}$ and $1 \times 10^{-4} \text{ M}\, 9$-AA, respectively.

The voltage dependence of pulsing recovery from the conditioned block was evaluated with the pulse sequence shown in the inset of Fig. 7 B. The peak
current associated with the test pulse was normalized to the one following conditioning pulses to -10 mV, where the maximum recovery occurred. Fig. 7 B shows that recovery is enhanced by pulsing to relatively low depolarizations, which could open the m gate of the sodium channel but do not exhibit the voltage-dependent inhibition by themselves (see Fig. 6 A).

**RECOVERY FROM FREQUENCY-DEPENDENT BLOCK IS VOLTAGE DEPENDENT, BUT INDEPENDENT OF CURRENT** A question arises as to whether this maximum recovery is the result of the inward flowing sodium ions which knock the 9-AA molecule out of the blocking site. An experiment was designed to test this notion by varying the external sodium ion concentration. An axon bathed in an external solution containing 50 mM Na and internally perfused with a solution containing 50 mM Na had a reversal potential of approximately 0 mV. The sodium current was small at the potentials around 0 mV, whereas the inward sodium current was large at 0 mV for an axon bathed in 450 mM external sodium. However, Fig. 7 B shows that the voltage dependence of recovery from 9-AA block is clearly the same for axons bathed in either high (filled circles) or low (open circles) sodium media. These results suggest that the recovery from 9-AA block is current independent, but voltage dependent.

**Na Inactivation and Frequency-Dependent Block**
Both conventional double-pulse technique (Hodgkin and Huxley, 1952a) and repetitive stimulation using trains of conditioning pulses combined with test pulses (Courtney, 1975) were used to obtain the potential dependence of the Na inactivation in the presence of 9-AA. No appreciable shift in the curve relating the Na inactivation to potential was observed with either method.

**Pronase Treatment Removes the Frequency-Dependent Inhibition but Does Not Affect the Steady-State Voltage-Dependent Inhibition**
In the pronase-treated axon, 9-AA exhibits many features that are different from those observed in the axon with an intact inactivation mechanism. Among these differences, it is most interesting to see that pronase treatment removes the frequency-dependent block caused by 9-AA.

**FREQUENCY-DEPENDENT BLOCK BY 9-AA DISAPPEARS AFTER PRONASE TREATMENT**
The frequency-dependent block of the sodium channel by 9-AA was almost completely eliminated after removal of sodium inactivation by internal perfusion of pronase. Because of removal of the inactivation mechanism, the block of the sodium channel by the 9-AA molecule is essentially complete at the end of a single 8-ms step depolarization. Upon repolarization, the m gate of the channel cannot be closed until the channel is free of the 9-AA molecule. This is reflected in the slow decay of the sodium tail current upon repolarization (see inset of Fig. 8). The double-pulse technique as shown in Fig. 8 demonstrates that the recovery from the time-dependent inhibition follows a single exponential time-course with a time constant of 0.75 ms. Thus, the change of a drug-bound channel to a drug-free channel proceeds with a time constant of 0.75 ms during repolarization. The consequence of a modification of the m gate by 9-AA is twofold: First, no cumulative frequency-dependent block is observed provided that the pulsing frequency is less than several tens of hertz; second, the voltage-
dependent block observed with multiple conditioning pulses (see Fig. 6 B) does not reveal the true voltage dependence because the "lock in" mechanism provided by the h gate has been removed by pronase treatment.

**9-AA induces a time-dependent block of the sodium current** 9-AA-induced decline of the sodium current follows a single exponential time-course (Fig. 9) in pronase-treated axons. The time constant decreased with increasing concentration of 9-AA. For example, the time constants at +80 mV were 2.0 ± 0.18 and 1.0 ± 0.19 ms, respectively, for 3 × 10⁻³ and 1 × 10⁻⁴ M 9-AA.

![Figure 8](image_url)

**Figure 8.** Recovery from the time-dependent inhibition by 0.1 mM 9-AA in pronase-treated axons. Double pulses separated by intervals of Δt were applied to measure the recovery from time-dependent inhibition which occurred during the first pulse. The difference in peak sodium currents during the first pulse (I₁) and that during the second pulse (I₂) is normalized and plotted semilogarithmically as a function of intervals. Recovery from time-dependent inhibition follows a single exponential time-course with a time constant of 0.75 ms.

**The steady-state block remains voltage-dependent after pronase treatment**

The steady-state inhibition of the sodium conductance by 9-AA at various voltages was estimated at the end of 8-ms step depolarizations. This steady-state inhibition clearly shows a voltage dependence, being more pronounced at more positive potentials. This kind of voltage-dependent inhibition was also observed with 9-AA in the h-gate intact axon (Fig. 6 A). A comparison between these two experimental conditions is shown in Fig. 6 A and B. In the pronase-treated axon, steady-state inhibition could be achieved with a single-step depolarization to +80 mV, whereas in the h-gate intact axon, steady-state inhibition could only be achieved with 20 multiple pulses (at 1 Hz). When curve I is compared to curve IV (in Fig. 6), it is clear that the steady-state inhibition by 9-AA in the h-gate intact axon was slightly less than that observed in the pronase-treated axon.
The binding constants used in fitting these two curves: $K_a$ was estimated to be $1.2 \times 10^4 \text{M}^{-1}$ and $1.7 \times 10^4 \text{M}^{-1}$ for curves I and IV, respectively.

**The Voltage Dependence of 9-AA Block in Pronase-Treated Axon**

In the pronase-treated axon, 9-AA block of Na channel was voltage dependent. The voltage dependence could be accounted for in terms of a simple two-barrier model adopted from Woodhull's model for ionic block of Na channels (Woodhull, 1973). The sodium currents in the pronase-treated axon before and during internal application of $1 \times 10^{-4} \text{M}$ 9-AA are shown in Fig. 10 A and B, respectively. The amplitudes of sodium currents were measured at the peak or at the end of an 8-ms step depolarization and plotted as a function of the membrane potential (Fig. 10 C). The control current-voltage relation is essentially the same as that of the normal axon whether it was obtained at the peak or at the end of the pulse. In the presence of 9-AA, the current-voltage relation ($\Delta$) looked rather normal if the sodium currents were measured at the peak. The peak conductance was suppressed but the $I-V$ relation was still linear.

![Diagram](https://example.com/diagram.png)

**Figure 9.** Time-dependent block of Na currents by 9-AA in pronase-treated axons. In A and B the upper currents are associated with depolarizations to $+80 \text{mV}$ and the lower currents associated with depolarizations to $0 \text{mV}$. 9-AA induces a time-dependent decline of both inward and outward sodium currents in a manner that depends upon the internal concentration of 9-AA.

However, if the currents were measured at the end of 8-ms pulses the current-voltage relation ($\bigcirc$) was affected by 9-AA in two ways: first, the amplitude of the sodium current was suppressed; second, the suppression was enhanced with increase in depolarization. In other words, 9-AA blocks the sodium current in a manner dependent on the membrane potential. Because the reversal potential for sodium channels is not affected, the decrease in the sodium current must be due to the decrease in the sodium conductance.

The voltage-dependent decrease in the sodium conductance suggests the site of action of 9-AA is within the membrane. Accumulating evidence has indicated that 9-AA, as well as many other ionic channel blockers, may directly block Na channels through their binding to a site within Na channel, thus "plugging up" the channel.

Following Woodhull's treatment of the voltage-dependent block by $\text{H}^+$ ion of the Na channel (Woodhull, 1973), a two-barrier model was constructed. The inner barrier represents the one over which the cationic form of 9-AA has to
cross from the axoplasmic side in order to bind inside the channel. The cationic form of 9-AA cannot, however, hop over the second barrier, an insurmountable barrier. Table II shows that the binding site is located halfway down the membrane field in the channel. The dissociation constant at 0 mV is estimated to be $6 \times 10^{-5}$ M. The actual fit of the current-voltage relation in the presence of $1 \times 10^{-4}$ M 9-AA is shown in Fig. 10 C. The fit by Eq. 1 is quite satisfactory (indicated as dotted line). At this concentration of 9-AA, a 5-mV shift in conductance-voltage relation toward depolarization considerably improves the fit at the membrane potential more negative than $-20$ mV, where the opening of the sodium channels is very sensitive to the membrane potential (not shown in the figure).

**Frequency-Dependent Block Did Not Occur in Pronase-Treated Axons with a Simulated Inactivation of Na Conductance**

In a pronase-treated axon, a time-dependent decline of the sodium conductance can be induced by an internal application of pancuronium (Yeh and Narahashi, 1977). Fig. 11 A shows that the sodium current associated with a step depolarization to 80 mV rises quickly and fails to inactivate after pronase treatment.

**Figure 10.** Voltage-dependent block of Na conductance by internal perfusion of $1 \times 10^{-4}$ M 9-AA in a pronase-treated axon. Families of Na current of pronase-treated axon before (A) and during (B) treatment with $1 \times 10^{-4}$ M 9-AA. Internal solution contains 20 mM TEA. The axon was held at a holding potential of $-80$ mV and depolarized by test pulses ranging in 20-mV steps from $-60$ to $+80$ mV. In C, the steady-state values for the control (●) and in the presence of 9-AA (○) are measured 8 ms after initiation of step depolarization and plotted as a function of the membrane potential. Na currents measured in the presence of 9-AA were scaled down using Eq. 1 as described in the text and denoted as the broken lines in the figure. The peak Na currents in the presence of 9-AA were also measured, and the current-voltage relation (Δ) is rather linear at the potentials more positive than 0 mV. The curves for measured values (● and Δ) were fitted by eye.
After the internal application of $1 \times 10^{-4}$ M pancuronium, the sodium current rises normally, but starts to decline as depolarization continues. Phenomenologically, the pancuronium-induced decline in sodium conductance resembles that caused by the sodium inactivation mechanism (h gate). Mechanistically, however, they differ from each other (Yeh and Narahashi, 1977; Yeh and Armstrong, 1978). The decline in the sodium conductance in the presence of

### Table II

VALUES OF THE PARAMETERS FOR 9-AA BLOCKAGE OF SODIUM CHANNELS OF PRONASE-TREATED AXONS*

| Axons          | Concentration | $M$  | $K_d$ |
|----------------|---------------|------|-------|
| 7-9-75A        | 30            | 0.50 | 50    |
| 7-9-75A        | 100           | 0.50 | 40    |
| 7-10-75A       | 30            | 0.50 | 110   |
| 7-10-75B       | 100           | 0.52 | 50    |
| 7-11-75B       | 30            | 0.48 | 60    |
| Mean ± SD      | 0.50 ± 0.01   | 62 ± 27.7 |

* The Woodhull model for $H^+$ blockage of Na channels (Woodhull, 1973) is modified to account for 9-AA blockage which is assumed to occur exclusively from the axoplasmic side. 9-AA can enter the sodium channel from the axoplasmic side but cannot go through because of its bulky size.

† Fraction of the electrical distance across the membrane from the inside.

§ The apparent dissociation constant at 0 mV.

---

pancuronium is more likely the result of the block (or occlusion) of the open channel by the drug molecule, whereas the decline of the sodium conductance of the normal perfused axon is due to closure of the channel by the inactivation mechanism (h gate).

Internal application of $1 \times 10^{-4}$ M 9-AA to the pancuronium-treated axon did not exhibit the frequency-dependent inhibition of the sodium channel (Fig. 11
An internally applied pancuronium could simulate an inactivation of sodium conductance, but failed to "reconstitute" the frequency-dependent block caused by 9-AA. This result reveals the importance of the \( h \) gate in controlling the "openness" of the sodium channel and in modulating the binding of the drug molecule with the sodium channel.

**9-AA Also Affects Potassium Channels**

9-AA also blocked potassium channels, which differed significantly from its effect on the sodium channel with intact \( h \) gate. During the rising phase of the potassium current, 9-AA exhibited some frequency-dependent removal of block of potassium channels, which was similar to aminopyridines block (Yeh et al., 1976). At the late phase of the potassium current, however, 9-AA block of K channels is in many ways reminiscent of its block of Na channels of the pronase-treated axon, namely, the time dependence and the voltage dependence. It is interesting to note that TEA derivatives also cause the time- and voltage-dependent block of the potassium channel (Armstrong, 1969, 1971). It appears that 9-AA exhibits the characteristics of block shared by both aminopyridines and tetraethylammonium derivatives.

**Stop-Flow Experiment**

The perfusion rate of internal solution was varied while the axon was bathed in an external solution containing 9-AA. Fig. 12 B1 and B2 shows that stopping internal perfusion enhances the 9-AA block of the sodium and potassium currents. The 9-AA block is rapidly reversed by resuming internal perfusion with drug-free medium (Fig. 12 C1 and C2). Families of membrane currents shown in Fig. 12 C1 and C2 look almost the same as those of the controls in Fig. 12 A1 and A2. In the absence of drug, stopping the flow of internal solution did

---

**Figure 12.** Relief of externally applied 9-AA block by internal perfusion with drug-free solution. 9-AA was applied externally to an internally perfused axon while suspending the internal flow (B1 and B2) or after resuming the internal flow (C1 and C2). Upper records, experiments in ASW; lower records, experiments in ASW added with 300 nM TTX. Families of ionic currents were taken 10 min after changing the solution or changing the internal perfusion rate.
not have any appreciable effect on the Na or K currents. The effect of 9-AA on the potassium current can be seen more clearly in an axon bathed in ASW containing 300 nM TTX. The lower part of Fig. 12 shows the result of such an experiment.

When the internal application of 9-AA was compared to its external application, for the same duration of exposure, the effect of 9-AA was more pronounced with internal application than with external application. This differential "sideness" effect exerted by internal and external applications of 9-AA and the result with stop-flow experiments strongly suggest that 9-AA acts from the axoplasmic side of the membrane in inhibiting both the sodium and the potassium channels.

DISCUSSION
The 9-AA block of Na channels can be divided into three phases: the initial block, the resting block, and the conditioned block. The initial block can be observed only during the first pulse. The resting block appears after several minutes of rest after the conditioned block. The conditioned block exhibits a frequency and a voltage dependence in the axon with the intact Na inactivation h gate (Hodgkin and Huxley, 1952b). Pronase treatment removes the frequency-dependent block but does not affect the voltage dependence of the steady-state block. In pronase-treated axons, a single 8-ms pulse is required for the block at each voltage to reach a steady state which can only be achieved after multiple pulses in the axon with intact h gate.

Conceptual Model for Voltage- and Frequency-Dependent Block
9-AA block of Na channels is interpreted with a hypothesis based on the following assumptions: (a) The 9-AA molecule binds to a site in Na channel when the channel is open, and the binding reaction is voltage dependent. (b) The 9-AA-bound channel will not conduct ions. (c) The 9-AA-bound channel can be inactivated by closing of the h gate as the normal channel. (d) Recovery from block is fast in the pronase-treated axon and slow in the normal axon, when axons are held at rest potentials. The justification and interpretation of the above assumptions are discussed in detail in the following sections.

VOLTAGE-DEPENDENT BLOCK OF NA CHANNEL BY 9-AA
The voltage-dependent block by 9-AA in the pronase-treated axon can be accounted for in terms of a simple two-barrier model adopted from Woodhull's model for ionic block of sodium channels (Woodhull, 1973). In this model, the main pathway for 9-AA to bind to the sodium channel is assumed to be via the hydrophilic pathway from the axoplasmic side of the membrane because 9-AA, having a pKa of 9.9, exists almost entirely in the cationic form at internal pH 7.3. In other words, the open sodium channel is visualized as the main pathway through which the cationic form of the molecule gains access to or leaves the binding site inside the channel. The important implication of this model is that the gating of Na channels is capable of modulating the drug-receptor interaction. By using a model based on a single binding reaction which is voltage dependent, a resting inhibition and frequency- and voltage-dependent inhibition could be reproduced in an h-gate intact axon.
The following discussion is principally concerned with the mechanism of how the frequency dependence is brought about by the activation mechanism of m gate and the inactivation mechanism of h gate.

**H Gate Modulates Drug-Na Channel Interaction**

The h gate is capable of modulating the drug-channel interaction in three ways. The first two ways occur during depolarization and the last one occurs during the prepulse potential. First, the closing of the h gate decreases the number of Na channels available for opening, and this would limit the access of the drug molecule to the binding site inside the channel. Second, once the drug molecule is bound, Na inactivation of the drug-bound channel proceeds as a normal channel, the closure of the gate of the drug-bound channel preventing the drug molecule from leaving the channel. In other words, the drug molecule is trapped inside the Na channel. Thus an inhibition associated with each conditioning pulse is locked in and the cumulative effect occurs upon repetitive pulsing. These two processes of modulating drug-channel interaction occur during depolarization. Fig. 5 shows that multiple conditioning short pulses are more effective in enhancing 9-AA block of Na current than a single 30-ms pulse although the total duration of depolarization pulse is the same. Similar results have been reported on QX-314 block in nodes of Ranvier (Strichartz, 1973). These results not only support the first point mentioned above, but also indicate that the total open time of Na channels is more important than the total duration of depolarizing pulse in determining the degree of block. However, the result of another set of experiments, in which the inactivation of pronase-treated Na channels is artificially simulated by internal application of pancuronium, shows that 9-AA loses its frequency-dependent block (see Fig. 11). These results suggest that the h gate is essential for the frequency-dependent block to occur because the inactivation of h gate provides the lock-in mechanism. However, it should be emphasized here that presence of the h gate does not increase the affinity of Na channels for 9-AA. The third way for the h gate to modulate the drug-channel interaction takes place during the prepulse potential because the prepulse potential controls the degree of Na channel activation during a test pulse, and a channel in activated (open) conformation is a prerequisite for drug molecule and channel interaction to occur. Thus the onset of frequency-dependent inhibition is directly related to the degree of the availability of Na channel for activation (Fig. 4 C). Shifting of the voltage dependence of Na inactivation in the hyperpolarizing direction by a drug has been proposed to explain the use-dependent onset or removal of inhibition of Na channels by GEA-968 (Courtney, 1975). The voltage dependence of Na inactivation, determined by the conventional two-pulse method of Hodgkin and Huxley (1952a) or by a rapid train of prepulse-test pairs used by Courtney (1975), is not shifted to an appreciable extent by 9-AA treatment.

Slow sodium inactivation induced by changing the holding potential or by a prepulse lasting a second or more seems to play an important role in modulating the kinetics of frequency-dependent block of Na channels in nodes of Ranvier caused by tertiary amine local anesthetics (Khodorov et al., 1976; Hille, 1977). It remains to be seen whether this slow sodium inactivation affects the kinetics of 9-AA block of Na channels.
M Gate Also Modulates Drug-NA Channel Interaction

The cationic form of local anesthetics is thought to interact with Na channels only when the channels are open. That is, the opening of an m gate is prerequisite for the occurrence of 9-AA block. Two observations support this idea: First, 9-AA induces a time-dependent block of Na conductance in the pronase-treated axon without affecting the rising phase of sodium conductance; second, 9-AA block of Na conductance is relatively small without previous subjection to any depolarizing pulse that might open the channel. The involvement of the m gate in modulating the drug-Na channel interaction during depolarization has been emphasized often (Strichartz, 1973; Courtney, 1975; Hille, 1977). However, the importance of the m gate in modulating the drug-channel binding during hyperpolarization to negative membrane potentials has been overlooked. Normally, hyperpolarization to a negative membrane potential will close the m gate, which is evidenced in the fast sodium tail current. In the presence of the drug molecule, however, the m gate may be affected. The m gate of the 9-AA-bound channel becomes immobilized and, therefore, cannot be closed upon repolarization until the channel is free of the 9-AA molecule. The slowness of decay of tail current upon repolarization to $-80 \text{ mV}$, as shown in Fig. 8, supports this notion. This slowness has also been observed with internal application of pancuronium, and it is interpreted as a consequence of the slow rate of dissociation of pancuronium from the Na channel (Yeh and Narahashi, 1977).

In the pronase-treated axon, the m gating upon repolarization plays an important role in modulating the 9-AA-Na channel interaction. Inasmuch as the m gate of the 9-AA-bound channel apparently cannot be closed upon repolarization and the lock-in mechanism of the h gate has been removed by pronase treatment, the 9-AA molecule is free to leave the channel during the repolarization. The newly freed channel becomes conducting to Na$^+$ ions and is then subject to closure by the m gate. Thus repolarization shifts all 9-AA-bound channels to drug-free channels, and this reestablishes the initial condition for the next depolarization. The consequence of this interaction between binding reaction and gating mechanism in a pronase-treated axon is that there is no frequency-dependent block. That is, repetitive pulsing does not enhance the block. This also explains the observation that a single, long depolarizing pulse is more effective than multiple short pulses to enhance this block in the pronase-treated axon. In contrast to the pronase-treated axon, multiple short pulses are more effective in producing the 9-AA block than a single long pulse when the h gate is intact.

If the m gate of the drug-bound channel in an axon with intact h gate behaved the same way as that in the pronase-treated axon, recovery from block should occur within a few milliseconds, and there should be no frequency-dependent block at 1 Hz, because Na inactivation recovers with a time constant of a few milliseconds at $-80 \text{ mV}$ and the drug-bound channel is free of 9-AA with a time constant of 0.75 ms (see Fig. 8). But resting recovery from the conditioned block at $-80 \text{ mV}$ required approximately 20 s (Fig. 7 A). There are two possibilities to account for this slow recovery. One possibility is that 9-AA induces a slow inactivation which recovers with a time constant of 20 s.
Alternatively, the m gate of the 9-AA-bound channel may close and 9-AA thus may be trapped in the channel. Then the slow recovery is probably due to random opening of channels at rest and 9-AA can dissociate from the open channel. The observation that repetitive pulsing to −20 mV accelerated the recovery (Fig. 7 A) seems to argue against the first possibility, and it supports the alternative hypothesis.

**Previous Models Concerning Frequency- and Voltage-Dependent Block**

The voltage-dependent inhibition of sodium currents of nodes of Ranvier by QX-314 was first described in detail by Strichartz (1973). He proposed a model based on the observation that QX-314 molecules interact with open sodium channels from the axoplasmic side and bind within these channels, causing block. The equilibrium constant for drug binding to an open channel was proposed to be voltage dependent.

The degree of 9-AA induced steady-state block obtained by a multiple conditioning pulse schedule in an axon with intact h gate is similar to that obtained by a single long pulse in the pronase-treated axon (see Fig. 6). This result seems to justify Strichartz's (1973) assumption that the voltage-sensitive steady-state inhibition of Na currents by QX-314 in nodes of Ranvier obtained by the multiple conditioning pulse technique represents the equilibrium between QX-314-bound and unbound Na channels.

The voltage dependence of block obtained by both multiple conditioning pulse techniques in the h-gate intact axon (see Fig. 6 A) and single-pulse technique in the pronase-treated axon (Figs. 6 B and 10 C) is fitted fairly well using a modified Woodhull's model (1973). In the present conceptual model the interaction between the voltage-dependent drug-receptor binding reaction and the gating machinery of the sodium channel manifests itself as resting inhibition and voltage-dependent inhibition depending on the conditioning pulse wave forms. Here, the resting (or tonic) inhibition is treated as a transient phenomenon but not as an equilibrium state. In this respect, the present conceptual model is different from Strichartz's model (1973).

Strichartz's model was extended by Courtney (1975). He explained the frequency- (or use-) dependent onset or removal of inhibition of Na channels by GEA-968 in terms of a shift of voltage dependence of Na inactivation in a hyperpolarizing direction. This shift in voltage dependence of Na inactivation will further enhance the voltage-dependent block as described by Strichartz (1973). The shift itself, however, does not explain the voltage dependence of block occurring at the conditioning potentials more positive than −20 mV because the h values reach a steady-state level, which is zero in nodes of Ranvier and is between 0.1 and 0.2 in internally perfused squid axons.

The voltage-dependent block as seen with QX-314 in nodes of Ranvier or with 9-AA in squid axons can most likely be attributed to the voltage dependence of the binding reaction itself. This notion is substantiated by experiments with 9-AA in pronase-treated axons. The voltage-dependent block by 9-AA remains intact even though the sodium inactivation mechanism has been removed by pronase treatment. Thus, the h gate itself is probably not required for voltage-
dependent binding of 9-AA to the receptor, but it merely modulates the binding indirectly to produce the block which depends upon the frequency of pulsing. However, in the case of QX-314, the voltage and frequency dependence of block disappears suggesting that the h gate is itself directly involved in the binding of QX-314 with Na channels, which is frequency and voltage dependent only when the h gate is intact (Yeh, 1978).

The modulated receptor model of Hille (1977) is, in general, adequate to account for the frequency- and voltage-dependent block of ionic channels caused by local anesthetics. Detailed kinetic analysis of drug-channel interaction is yet to be carried out. In view of the recent studies of Mrose and Ritchie (1978), however, the hypothesis that there exists a single specific receptor for all local anesthetics remains to be tested.

In the above discussion, I have emphasized the importance of gating machinery of Na channels in modulating the drug-Na channel interaction which manifests itself as resting inhibition, frequency-, and voltage-dependent inhibition. Further studies of ionic blockage with various local anesthetics may reveal the nature of receptor (or binding site) and the architecture of ionic channels.

I would like to thank Dr. Toshio Narahashi for support and encouragement at all stages of this work. Thanks are also due to Chuck Parker and Denise Ring for data analysis, and Amy Bicego, June Canzoneri, and Deborah Stosich for secretarial assistance.

This work was supported by U.S. Public Health Service grants NS 14144, GM 24866, and GM-00442. The experiments were performed at the Marine Biological Laboratory, Woods Hole, Mass.

Received for publication 13 December 1977.

REFERENCES

ALBERT, A. 1966. The Acridines. St. Martin's Press, New York. 2nd edition. 155.

ARMSTRONG, C. M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. J. Gen. Physiol. 54:553-575.

ARMSTRONG, C. M. 1971. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. J. Gen. Physiol. 58:413-437.

ARMSTRONG, C. M., F. BENZANILLA, and E. ROJAS. 1973. Destruction of sodium conductance inactivation in squid axons perfused with pronase. J. Gen. Physiol. 62:375-391.

BAKER, P. F., A. L. HODGKIN, and T. I. SHAW. 1961. Replacement of the protoplasm of a giant nerve fibre with artificial solutions. Nature (Lond.). 190:885-887.

CAHALAN, M. D., and B. I. SHAPIRO. 1976. Current and frequency dependent block of sodium channels by strychnine. Biophys. J. 16 (2, pt. 2): 76a. (Abstr.).

COURTNEY, K. R. 1975. Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. J. Pharmacol. Exp. Ther. 195:225-236.

HILLE, B. 1977. Local anesthetic: hydrophilic and hydrophobic pathways for the drug-receptor reaction. J. Gen. Physiol. 69:497-515.

HODGKIN, A. L., and A. F. HUXLEY. 1952a. The dual effects of membrane potential on sodium conductance in the giant axon of Loligo. J. Physiol. (Lond.). 116:497-506.

HODGKIN, A. L., and A. F. HUXLEY. 1952b. A quantitative description of membrane
current and its application to conductance and excitation of nerve. J. Physiol. (Lond.). 117:500-544.

Khodorov, B. I., L. D. Shishkova and E. M. Peganov. 1976. The effect of procaine and calcium ions on slow sodium inactivation in the membrane of Ranvier's node of frog role of sodium inactivation. Biochim. Biophys. Acta. 433:409-435.

Mrose, H. E., and J. M. Ritchie. 1978. Local anesthetics: do benzocaine and lidocaine act at the same single site? J. Gen. Physiol. 71:223-225.

Strichartz, G. R. 1973. The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. J. Gen. Physiol. 62:37-57.

Taylor, R. E. 1959. Effect of procaine on electrical properties of squid axon membrane. Am. J. Physiol. 196:1071-1078.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. J. Gen. Physiol. 61:687-708.

Wu, C. H., and T. Narahashi. 1973. Mechanism of action of propanolol on squid axon membrane. J. Pharmacol. Exp. Ther. 184:155-162.

Yeh, J. Z. 1978. Sodium inactivation mechanism modulates QX-314 block of sodium channels in squid axons. Biophys. J. 24:569-574.

Yeh, J. Z., and C. M. Armstrong. 1978. Immobilization of gating charge by a substance that simulates inactivation. Nature (Lond.). 273:387-389.

Yeh, J. Z., and T. Narahashi. 1975. Characterization of sodium channel block by 9-aminoacridine. 5th International Biophysical Congress, Copenhagen. p. 133.

Yeh, J. Z., and T. Narahashi. 1976. Frequency-dependent block of sodium channel in normal and pronase-treated squid axons. Fed. Proc. 35(3):846.

Yeh, J. Z., and T. Narahashi. 1977. Kinetic analysis of pancuronium interaction with sodium channels in squid axon membranes. J. Gen. Physiol. 69:293-323.

Yeh, J. Z., G. S. Oxford, C. H. Wu, and T. Narahashi. 1976. Interactions of aminopyridines with potassium channels of squid axon membranes. Biophys. J. 16:77-81.