Interaction of n-Alkylguanidines with the Sodium Channels of Squid Axon Membrane

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ABSTRACT The effects of n-alkylguanidine derivatives on sodium channel conductance were measured in voltage clamped, internally perfused squid giant axons. After destruction of the sodium inactivation mechanism by internal pronase treatment, internal application of n-amylguanidine (0.5 mM) or n-octylguanidine (0.03 mM) caused a time-dependent block of sodium channels. No time-dependent block was observed with shorter chain derivatives. No change in the rising phase of sodium current was seen and the block of steady-state sodium current was independent of the membrane potential. In axons with intact sodium inactivation, an apparent facilitation of inactivation was observed after application of either n-amylguanidine or n-octylguanidine. These results can be explained by a model in which alkylguanidines enter and occlude open sodium channels from inside the membrane with voltage-independent rate constants. Alkylguanidine block bears a close resemblance to natural sodium inactivation. This might be explained by the fact that alkylguanidines are related to arginine, which has a guanidino group and is thought to be an essential amino acid in the molecular mechanism of sodium inactivation. A strong correlation between alkyl chain length and blocking potency was found, suggesting that a hydrophobic binding site exists near the inner mouth of the sodium channel.

INTRODUCTION

Much of our knowledge about the nature of the ionic channels responsible for the generation of nerve impulses has been gained through the study of the interaction of blocking agents with these channels. A good example of this type of approach is Armstrong's study (1971) of a series of quaternary ammonium derivatives that block potassium channels of nerve membrane. He showed that potassium channels have a relatively nonselective inner mouth associated with a hydrophobic region. Similarly, sodium channels have been probed using channel blockers such as local anesthetics and tetrodotoxin (TTX) (reviewed by Ritchie, 1979; Narahashi, 1974). In many previous studies small charged molecules such as local anesthetics (Taylor, 1959; Narahashi et al., 1969; Strichartz, 1973; Courtney, 1975; Hille, 1977), strychnine (Shapiro, 1977; Cahalan and Almers, 1979), 9-aminoacridine (Cahalan, 1978; Yeh, 1979), and pancuronium (Yeh and Narahashi, 1977; Yeh and Narahashi, 1977; Yeh and
Armstrong, 1978) have been shown to block sodium channels and to interfere with the channel gating mechanism. In addition, several blocking agents have been found to substitute for the natural sodium inactivation mechanism in axons treated with pronase to remove the inactivation gate. Among them are polyglycylarginine (Eaton et al., 1978) and quaternary ammonium derivatives (Rojas and Rudy, 1976). These and other studies have led to our present concept of the sodium channel as an aqueous pore whose gating structure can serve as a site for drug interaction.

The guanidinium group has been implicated as a channel blocker in several ways. First, it appears to be the functional group responsible for the block of sodium current in axons by TTX and saxitoxin (STX) (Kao and Nishiyama, 1965; Hille, 1975). Second, although guanidine itself can pass through the sodium channel, methylguanidine cannot (Hille, 1971; Hironaka and Narahashi, 1977). Third, the amino acid arginine, whose guanidino group is positively charged at physiological pH, appears to be necessary for the normal functioning of the sodium inactivation process (Rojas and Rudy, 1976; Eaton et al., 1978). Finally, studies in our laboratory on frog skeletal muscle have shown that n-alkylguanidine derivatives block ionic channels of endplate (Farley et al., 1979; Watanabe and Narahashi, 1979). Thus, n-alkylguanidines provide an excellent opportunity to study the gating mechanism of nerve membrane sodium channels. In the present study, internally applied n-alkylguanidines have been found to block sodium channels through binding to the open channels. The effectiveness of these compounds in blocking channels was enhanced by increasing the alkyl chain length, suggesting hydrophobic interaction with the binding site.

**MATERIALS AND METHODS**

Giant axons isolated from the squid, *Loligo pealei*, obtained at the Marine Biological Laboratory, Woods Hole, Mass., were internally perfused by the roller method originally developed by Baker et al. (1961) and modified by Narahashi and Anderson (1967). The axons were mounted in a Plexiglas chamber, perfused externally with artificial seawater (ASW), and voltage clamped by the axial wire method described previously (Wu and Narahashi, 1973). The response time (10-90% of a step-voltage pulse) under voltage-clamp conditions was ~10 μs. Leakage currents and capacitative currents were electronically subtracted from the membrane currents, assuming linear current-voltage (I-V) relationships. Approximately two-thirds of the series resistance was compensated by a feedback circuit. The holding potential was either −80 or −90 mV. Membrane potential measurement was corrected for junction potentials.

Normal ASW had the following composition (mM): Na⁺, 450; K⁺, 10; Ca²⁺, 50; Cl⁻, 576; and HEPES buffer, 5. The final pH was adjusted to 8.0 with NaOH or HCl. In some experiments, K⁺ was left out and the Na⁺ concentration was reduced to either 225 to 150 mM by equimolar replacement with tetramethylammonium. The standard internal solution (SIS) had the following composition (mM): K⁺, 350; Na⁺, 50; glutamate⁻, 320; F⁻, 50; sucrose, 333; phosphate buffer, 15. The pH was adjusted to 7.3 with KOH or HCl. Many experiments were conducted with potassium-free internal solution, which had the following composition (mM): Cs⁺, 250; Na⁺, 50; glutamate⁻, 250; F⁻, 20; phosphate buffer, 15; sucrose, 400. The final pH was 7.3. In some experiments, pronase (0.3 mg/ml) was briefly added to the internal solution to
remove sodium inactivation (Armstrong et al., 1973). In all experiments, temperature was maintained constant at 8–10°C, as measured by a thermocouple mounted as close to the axon as possible in the central current-measuring electrode region.

The chemical structure of the n-alkylguanidine derivatives used in this study is shown in Fig. 1. These are amphipathic molecules with a guanidine group, which is positively charged at physiological pH, attached to a hydrocarbon chain of various lengths from one to eight carbons. The derivatives will be referred to by hydrocarbon chain length, i.e., C₁ for n-methylguanidine, C₂ for n-ethylguanidine, C₃ for n-propylguanidine, C₅ for n-amylguanidine, and C₈ for n-octylguanidine. C₂, C₅, C₆, and C₈ derivatives were synthesized by Dr. John Dutcher (National Cancer Institute, National Institutes of Health, Bethesda, Md.). C₁ was obtained from Sigma Chemical Co., St. Louis, Mo. Guanidine derivatives were always added to the internal perfusate, unless otherwise stated.

Membrane currents, recorded on film, were analyzed with the help of a digitizer linked to a programmable calculator (HP 986A digitizer and HP 9821 calculator, Hewlet-Packard Co., Palo Alto, Calif.). The output of the calculator, in the form of I-V curves, exponential least squares fit of the time-course of current decay, and kinetic model simulations of currents, was fed into a digital X-Y plotter (HP 9862A) to draw graphs.

![Figure 1. Structure of n-alkylguanidine.](image_url)

**RESULTS**

**Effects of n-Alkylguanidine Derivatives on Ionic Currents**

**INTERNAL APPLICATION** n-Alkylguanidine derivatives had a variety of blocking effects on sodium and potassium currents. Fig. 2 illustrates membrane currents recorded before and during internal perfusion of the test compounds. Depolarizing pulses to +100 mV and 0 mV were applied periodically until a steady-state effect was achieved, usually within 5 min. The differential effects of guanidine derivatives on sodium channels and potassium channels can be determined by comparing changes in peak transient currents with those in steady-state currents measured at 8 ms after the beginning of the pulse. Fig. 2 A illustrates the effect of the C₁ derivative (7 mM). The peak sodium currents were virtually unchanged. The steady-state potassium currents at +100 mV were reduced by 40%. Increasing the concentration of C₁ to 20 mM caused a 35% reduction of sodium current (not illustrated). Increasing the chain length by one carbon enhanced sodium channel block, as shown in Fig. 2 B, where it can be seen that the peak sodium currents in the presence of 7 mM C₂ were reduced by 40% at +100 mV. Further enhancement of block by increased hydrophobic chain length was shown by C₅ (Fig. 2C) and C₈ (not illustrated). The C₈ derivatives also followed this trend, but in addition, had
several other interesting features. Fig. 2 D shows that at +100 mV in the presence of C₈ (0.18 mM), both peak transient and steady-state currents were reduced by 50%, and that the time-course of the transient sodium currents was altered, as evidenced by the decreased time to peak. The steady-state potassium current was unaffected at 0 mV, but was reduced by 45% at +100 mV. The slope of the I-V relationship for steady-state potassium current in C₈-treated axons decreased with depolarization, indicating that the C₈ block of potassium channel was enhanced by depolarization.

In summary, derivatives with longer alkyl chains were more potent in blocking sodium and potassium channels. Longer chain length was correlated with shorter time to peak of the transient sodium currents and greater voltage-dependent block of the steady-state potassium currents. The rest of this paper will be devoted to the effects of n-alkylguanidines on sodium channels.

**EXTERNAL APPLICATION** The alkylguanidines were relatively ineffective in blocking sodium channels when applied externally. However, the effectiveness increased with increasing chain length. For example, C₅ at concentrations up to 1.0 mM had no effect when externally applied, whereas C₈ was somewhat effective at the same concentration. Fig. 3 shows an example of the effect of external application of 1.0 and 2.0 mM C₈ on the Na conductance-voltage relationship. C₈ at a concentration of 1.0 mM shifted the Na conductance-voltage relationship ~10 mV in the depolarizing direction without significantly changing maximum conductance. The reversal potential re-
mained constant throughout. C₈ at 2.0 mM reduced maximum conductance by ~43%. When the curve was normalized to take this factor into account, 2.0 mM C₈ was found to shift the conductance curve 16 mV in the depolarizing direction. Such a voltage shift can be explained by the development of a positive fixed charge resulting from the adsorption of C₈ to the outer surface of the membrane (Schafer and Rieger, 1974). The voltage dependence of sodium channel gating would then be altered in a manner similar to elevated external calcium concentration (Frankenhaeuser and Hodgkin, 1957). The fact that maximum conductance was also reduced suggests that some fraction of the sodium channels were blocked at the higher concentration.

![Graph showing effect of C₈ on conductance](image)

**Figure 3.** Effect of external application of C₈ on peak transient conductance of an internally perfused axon. External and internal solutions were ASW and SIS, respectively.

**Effects of Internally Applied n-Alkylguanidines on Sodium Conductance**

To examine in more detail the effects of n-alkylguanidine derivatives on sodium current, we eliminated potassium currents by replacing internal potassium with cesium. The external solution was also potassium free and usually had reduced sodium concentration. Under these conditions, sodium currents during an 8-ms test pulse decay with a single time constant (τᵢ) to a steady state (i.e., noninactivating) level, as shown in Fig. 4 A. Thus, there are two phases of sodium current, peak transient and steady state.

C₈ reduced steady-state sodium currents much more than peak sodium currents (Fig. 4 B). The fraction of current remaining after treatment with C₈
at several concentrations are listed in Table I. The half-blocking concentration was ~0.5 mM for peak currents and <0.1 mM for steady-state currents. Fig. 4 B also shows that, although the time-course of decay of sodium current was speeded up, the initial turn-on of sodium current was unchanged. The peak $I-V$ relationship for $C_5$ (in Fig. 4 C) shows that both inward and outward sodium currents were equally blocked and that the reversal potential was not changed, suggesting that the guanidinium block does not depend on the driving force for Na$^+$ and that the guanidine derivative does not alter sodium channel selectivity. The block was readily reversible upon washing with normal SIS.

The effect of $C_8$ on sodium conductance was nearly identical to that of $C_5$, except that $C_8$ was approximately 10 times more potent (see Table II). In contrast, the short-chain derivatives ($C_1$-$C_3$) were much less potent blockers and did not alter the time-course of sodium current decay during maintained depolarization.

The apparent facilitation of sodium inactivation by the long-chain derivatives can be explained by their direct action upon the h gate to speed its kinetics. A similar explanation has been advanced for the effects of octanol.
(Oxford and Swenson, 1979) and of the local anesthetic QX-314 (Yeh, 1978) on squid axons. Alternatively, guanidine derivatives may block open sodium channels in a time-dependent manner, as is the case for certain blocking agents (Yeh and Narahashi, 1977; Cahalan, 1978).

One way to detect drug interaction with sodium inactivation is by measurement of steady-state sodium inactivation ($h_a$) curve. C8 caused no shift in the $h_a$ plot along the voltage axis, but eliminated the foot of the curve at high depolarizations (Fig. 5 A). This effect can be explained by the previously described block of steady-state sodium currents. In contrast, a shift of 7 mV

| TABLE I | EFFECT OF C8 ON PEAK AND STEADY-STATE SODIUM CURRENT |
|---------|--------------------------------------------------------|
| Concentration | $I_{drug}/I_{control} (+100 \text{mV})^*$ |
| mM     |  |  |
| 0      | 1.0 | 1.0 |
| 0.1    | 0.8 | 0.2 |
| 0.3    | 0.6 | 0.1 |
| 1.0    | 0.4 | 0.1 |
| 3.0    | 0.2 | 0.1 |

* Averages of two experiments. Cumulative doses were applied.

| TABLE II | EFFECT OF C8 ON PEAK AND STEADY-STATE SODIUM CURRENT |
|----------|--------------------------------------------------------|
| Concentration | $I_{drug}/I_{control} (+100 \text{mV})^*$ |
| mM     |  |  |
| 0      | 1.0 | 1.0 |
| 0.005  | 0.9 | 0.4 |
| 0.010  | 0.7 | 0.1 |
| 0.050  | 0.6 | 0.1 |
| 0.100  | 0.5 | 0.1 |

* Averages of two experiments. Cumulative doses were applied.

in the hyperpolarizing direction was observed for the curve relating to the steady-state level of slow sodium inactivation ($s_a$) (Fig. 5 B). For this measurement, the membrane was held for 3 min at various potentials, stepped for 50 ms to $-80 \text{ mV}$ to remove the fast inactivation process, and then tested for the ability to produce peak sodium current with a short pulse to 0 mV. The observed shift is in the direction predicted by the adsorption of positive fixed charge at the inner surface of the membrane, but the fact that the $h_a$ curve was not shifted suggests that the effect on $s_a$ may reflect a tonic block of
sodium channels that is exerted only at holding potentials more positive than -70 mV, or the enhancement of slow sodium inactivation by the drug.

The apparent time-course of sodium inactivation measured from the falling phase of the sodium current could be described by a single exponential function (after subtraction of noninactivated current) in both control and drug-treated axons. Fig. 6 shows the dependence of the decay time constant on Cs concentration and membrane potential. The Cs derivative significantly reduced the time constant only at depolarizations greater than -20 mV and at concentrations in excess of 10 μM. However, these restrictions probably reflect the resolution limits imposed by the method of graphical analysis of the time-course, since, as Table II shows, even at 10 μM, steady-state sodium currents are reduced much more than peak currents, suggesting that sodium current decay indeed is facilitated, but not enough to be detected by our methods. The simplest explanation of these data is that n-alkylguanidines block sodium channels in a time-dependent manner.

**Figure 5.** Effects of internal application of 30 μM Cs on steady-state sodium inactivation (A) and slow inactivation (B). Pulse protocols are shown in insets. Peak sodium currents were normalized by dividing by the maximum current. Same axon in both A and B. External solution, K-free, 1/2-Na ASW; internal solution, K-free SIS plus Cs (open circles). Curves were drawn by eye.

Time-dependent Block of Sodium Channels

Sodium channels in axons in which sodium inactivation has been removed by internal perfusion of pronase can be blocked by Cs, as shown in Fig. 7 A and B. Current families were recorded after pronase treatment (Fig. 7 A) and after addition of 20 μM Cs (Fig. 7 B). Treatment with Cs, and to a lesser extent Cs, caused an apparent restoration of sodium inactivation by introducing a time-
dependent block of sodium current. The $I-V$ plot after pronase treatment (Fig. 7 C) shows that, whereas peak currents were only slightly decreased by $C_8$ application, steady-state sodium currents measured at the end of the 8-ms pulse were markedly reduced. This reduction in sodium conductance appears to be independent of voltage, as shown by the linearity of the $I-V$ curve in the range 0 to +100 mV. This was found in three of six axons. In the remaining axons, the steady-state $I-V$ plot bent slightly upward at high depolarizations.

(Fig. 6). Facilitation of sodium current decay by $C_8$ in an h gate-intact axon. Decay time constants determined from digitized current records plotted semilogarithmically against membrane potential and fitted with least squares line after subtraction of steady-state component. Correlation coefficient ($r$) was greater than 0.90 in all cases. External solution, K-free, 1/2-Na ASW; internal solution, K-free SIS. Holding potential was $-90$ mV.

(+70 to +100 mV). This effect was probably the result of deterioration of axons resulting from pronase and drug treatment, since it remained after the time-dependent block was reversed by washing with drug-free SIS.

The decay of sodium currents in pronase-treated axons after the application of $C_8$ followed an exponential time-course. Time constants of decay were determined from semilogarithmic plots and are expressed as a function of membrane potential in Fig. 8. The lack of steepness in the slope of these curves indicates that the time-course of block is only slightly voltage dependent. It
will be shown in a later section that if \( C_8 \) blocks open channels with voltage-independent rate constants, the time constant of decay will be slightly increased at potentials at which channel opening kinetics are slow. Thus, the fact that the time-course of block is nearly voltage independent agrees with the observation that the steady-state level of block is also voltage independent. In summary, the time-dependent, voltage independent block of sodium channels by \( n \)-alkylguanidines can account for their apparent facilitation of sodium inactivation.

These results suggest a simple model for sodium channel block whereby the guanidine derivative enters and occludes the open sodium channel in a manner similar to that of quaternary ammonium blocking agents such as pancuronium (Yeh and Narahashi, 1977). However, unlike pancuronium, \( n \)-alkylguanidines appear to bind near the mouth of the channel since the steady-state block is not affected by membrane potential. Another difference is that, in contrast to pancuronium, which causes a pronounced hook in sodium tail currents due to the interference of drug binding with \( m \) gate closure, the \( n \)-alkylguanidines do not affect tail current time-course. Fig. 9 shows tail currents elicited by repolarization at either the peak or the steady-state level of sodium current. The time-course of tail current decay was not significantly different from control records. In both pronase-treated and intact

**Figure 7.** Membrane currents associated with step depolarizations of 40 to 180 mV in 20-mV increments before (A) and after (B) internal application of 20 \( \mu \)M C\(_8\). (C) Peak sodium currents (circles) and steady-state sodium currents (squares) plotted as a function of membrane potential before (filled circles) and after (open symbols) application of C\(_8\). External solution, K-free 1/2-Na ASW; internal solution, K-free SIS.
axons, tail currents decayed with a single exponential time-course; no hook or prolongation was seen. The relationship between sodium conductance determined from tail current amplitude and the conditioning membrane potential can be used as another test of whether the guanidine block is voltage dependent, if we assume that the initial tail current amplitude directly measures current flow through unblocked channels. Fig. 10 shows that in a pronase-treated axon sodium conductance reached a saturated level at about 0 mV. After the application of 30 μM C₈, tail currents were measured either

![Graph showing decay time constant of sodium current in pronase-treated axons after internal application of C₈ in three axons.](image)

**Figure 8.** Time constant of decay of sodium current in pronase-treated axons after internal application of C₈ in three axons. For further details of analysis and experimental conditions, see legend of Fig. 6.

![Graph showing tail currents in a pronase-treated axon before (A) and after (B and C) internal application of C₈.](image)

**Figure 9.** Tail currents in a pronase-treated axon before (A) and after (B and C) internal application of C₈. Upper traces: membrane potential stepped briefly by conditioning pulse to +100 mV from a holding potential of -90 mV, then repolarized to -50 mV. External solution, (A) K-free 1/3-Na ASW and (B) K-free, full-Na ASW; internal solution, (A and B) K-free SIS.

upon repolarization at the peak sodium current or during the steady-state sodium current. The peak sodium conductance reached a constant level of 55% of control at about +20 mV and the steady-state conductance reached a constant level of 30% of control at -10 mV. Clearly, steady-state guanidine block is independent of membrane potential in the range of maximum sodium conductance. These tail current data suggest that block removal during repolarization does not significantly affect tail currents. Either m gate closure
is unaltered by the presence of blocker or the time-course of block removal is too slow to resolve by the method of tail current analysis, so that block removal upon repolarization cannot contribute measurably to tail current, even if the m gate is held open by the blocking ion.

**Figure 10.** Sodium conductance measured from tail currents in a pronase-treated axon before (open circles) and after (filled symbols) internal application of 30 µM Cs. Tail currents were elicited by repolarization to -50 mV when currents during the conditioning pulse had reached either peak (triangles) or steady-state levels (at 2 ms, circles). Conductance was determined by measuring tail currents at 100 µs from the start of repolarization and dividing by the driving force. Same axon as in Fig. 8.

**Figure 11.** Cumulative frequency-dependent block by internally applied 30 µM Cs in a h gate-intact (A) and a pronase-treated (B) axon. Outward currents elicited by a train of 10 pulses to +100 mV at five pulses per second. Inward currents were elicited after a rest interval of at least 10 s by a similar train of pulses to 0 mV. Currents elicited by the first pulses in each train are labeled a. Currents elicited by succeeding pulses are superimposed and are labeled b. Holding potential, -90 mV. External solution, K-free, 1/3-Na ASW; internal solution, K-free SIS plus Cs.

**Frequency-dependent Block**

A slow rate of block removal upon repolarization predicts that with repetitive stimulation the persistence of blocker in the channels will lead to cumulative
block. This phenomenon has been studied extensively with local anesthetics (Strichartz, 1973; Courtney, 1975; Hille, 1977; Cahalan, 1978; Yeh, 1979) and termed frequency- or use-dependent block. We have observed a similar phenomenon in axons treated with C₈ when stimulation frequencies exceeded two pulses per second. Fig. 11 shows examples of frequency dependence in both normal and pronase-treated axons. In these two experiments, axons treated with 30 μM C₈ were stimulated repetitively at five pulses per second to +100 and 0 mV. The largest currents resulted from the first pulse. The second and succeeding pulses produced smaller superimposed currents, presumably reflecting the saturated level of cumulative block. This level of block increased at higher pulse frequency. Pronase treatment left frequency dependence unaltered. Thus, unlike some local anesthetics (Cahalan, 1978; Yeh, 1979), interaction with the h gate is not the mechanism underlying frequency-dependent block by n-alkylguanidines. No cumulative effects were observed with C₃–C₅ at stimulation frequencies of 10 pulses per second or less.

Recovery from n-Alkylguanidine Block

The time-course of recovery from depolarization-induced inactivation in the presence of C₈ appears to be biphasic. The fast phase has the same time constant as recovery from sodium inactivation in a drug-free axon; and the other much slower phase probably represents the dissociation of drug molecules from the binding site in sodium channels.

The kinetics of recovery from frequency-dependent block, in an axon with normal sodium inactivation, were measured using the pulse protocol shown in the inset of Fig. 12. Sodium channels were first blocked to a steady-state level by pulsing 10 times to +80 mV at 4 Hz. Recovery from this conditioned block was monitored by a constant test pulse to 0 mV after various intervals between the last conditioning pulse and the test pulse. The sodium currents associated with the test pulses were normalized to the one after a 1-min pulse interval. These normalized data were plotted as a function of pulse interval (Δt).

Recovery from sodium inactivation in the absence of drug was the same as that determined by the conventional method; τᵣ was 3.5 ms at −80 mV. In the presence of C₈, the data were fitted with two exponential functions as follows: 1 − 0.6 exp (−t/τᵣ) − 0.4 exp (−t/τₑ), with τᵣ being 4.0 ms and τₑ 210 ms. Thus, in 30 μM C₈, repetitive pulsing at 4 Hz to +80 mV converted 40% of the sodium channels into nonconducting drug-bound channels, which recovered with a time constant of ~200 ms. Similar measurements in a pronase-treated axon showed that in the absence of normal inactivation, recovery from block followed a single exponential time-course with a time constant of 270 ms.

Correlation of Blocking Potency with Alkyl Chain Length

In pronase-treated axons, only C₅ and C₈ showed clear evidence of time-dependent block. This observation can be explained by the idea that the dissociation rate decreases with increasing chain length. Thus, for C₅–C₃ an equilibrium level of block is reached by the time of the peak sodium current, whereas C₅ and C₈ require several milliseconds to reach blocking equilibrium.
Accordingly, we determined the relationship between blocking potency and hydrocarbon chain length for C₁-C₃ on the basis of the reduction of peak sodium currents in h gate intact axons and for C₅ and C₆ on the basis of reduction of steady-state sodium currents in pronase-treated axons. We assumed a one-to-one interaction of n-alkylguanidine molecules with sodium channels in order to calculate the half-blocking concentrations (Kₐ). Fig. 13 shows that the increase in blocking potency can be fitted by the equation, 

\[ K_d = ae^{-bn} \]

where n is the number of -CH₂- groups, a = 187, and b = 1.2. The free energy (ΔG) per mole of -CH₂- for receptor-drug complex formation can be calculated according to the equation:

\[ \Delta G / -CH₂- = -RT \ln \frac{K_{n+1}}{K_n} = 650 \text{ cal/mol } -CH₂-. \]

**FIGURE 12.** Time-course of recovery from sodium inactivation (filled circles) and from block by internal application of 30 μM C₅ (open circles). Currents associated with test pulses were normalized by dividing by maximum current in the absence of conditioning depolarization. Pulse protocol shown in inset. Curves are fitted by a single exponential (control) or a double exponential (C₅) time-course. Holding potential, −80 mV.

**DISCUSSION**

This study has revealed the following points: (a) n-Alkylguanidines applied internally block sodium channels. Even at the highest concentration (2 mM) of the most potent derivative (C₅), the sodium current rose normally but with reduced amplitude and duration. This result can be interpreted by assuming that n-alkylguanidines block sodium channels only when they have been opened by depolarization. The steady-state block of sodium channels in the region of maximum conductance (at positive membrane potentials) is independent of membrane potential. This indicates a shallow penetration of the membrane field by the guanidinium head of the molecule. (b) Long-chain derivatives (C₅ and C₆) facilitate the apparent inactivation of sodium current,
and this effect can be explained, in part, by a time-dependent block. (c) At relatively high stimulus frequencies (i.e., greater than two pulses per second), Cs causes cumulative block, which is relieved under resting conditions (holding potentials, −70 to −100 mV) with a slow exponential time-course (200 ms). Both frequency dependence and the time-course of recovery were unchanged by pronase treatment, suggesting that the mechanism underlying these effects is unrelated to sodium inactivation. (d) The time-course of tail currents in h

Figure 13. Correlation of potency in blocking peak sodium current with hydrocarbon chain length. Apparent $K_{d}$ were calculated from the equation,

$$\frac{I_{Na, \text{drug}}}{I_{Na, \text{control}}} = \frac{K_d}{K_d + [\text{drug}]}.$$  

The least squares fit of the equation $n = a + b \ln K_d$ (where $n =$ number of carbons, $K_d =$ half-blocking concentration) gave $a = 4.5$, $b = -0.9$, with correlation coefficient $r = 0.99$. Data points are mean ± SD.

gate-intact or pronate-treated axons was unchanged by Cs. Probably only the channels unaffected by drug contributed to the tail currents. (e) There is a clear correlation of blocking potency with increasing alkyl chain length, which suggests an important hydrophobic component of the interaction of blocking molecule with receptor.

Hydrophobic interactions of chemical probes with ionic channels have been proposed by Armstrong (1971) and by Rojas and Rudy (1976) in squid axons, and by Reed and Trzos (1979) in eel electroplaques. Based on the observation
that the effectiveness of tetraethylammonium (TEA) derivatives increases with the substituted chain length, Armstrong (1971) proposed the presence of a hydrophobic binding site near the inner mouth of the potassium channel. Our results, together with those of Rojas and Rudy (1976) and Reed and Trzos (1979) strongly suggest that a hydrophobic binding site exists in the sodium channel.

Rojas and Rudy (1976) showed that in pronase-treated axons, internally applied long-chain TEA derivatives cause a time-dependent block of sodium channels very similar to our findings with n-alkylguanidines. Their estimate of $K_d$ for the TEA-C$_9$ derivative of 25 $\mu$M corresponds closely to our estimate of 23 $\mu$M for the guanidine-C$_8$ derivative. Also, their calculation of the change in free energy of complex formation ($\Delta G$) was 560 cal/mol, which agrees with our calculation of 650 cal/mol. Reed and Trzos (1979) found that n-alkylguanidines inhibit TTX binding to the sodium channels contained in membrane fragments isolated from electroplaques. They estimated the $K_d$ for the interaction of guanidine-C$_9$ and TTX to be 18 $\mu$M and the $\Delta G$ for this series to be 590 cal/mol. The close similarity of $K_d$ and $\Delta G$ values in these three sets of experiments suggests that the same binding site may be involved. However, the TTX binding site is on the external side of the membrane (Narahashi et al., 1967), whereas the channel blocking effects of TEA and guanidine derivatives were seen only with internal application. Also, the block by alkylguanidine derivatives was not strongly voltage dependent, suggesting that alkylguanidines do not enter deeply into the channels. The experiments with electroplaque membrane fragments allowed equilibration of alkylguanidines with both sides of the membrane. Thus, there may be an allosteric interaction between the internal guanidine binding site and the external TTX binding site. No study has been reported on the effects of long-chain TEA derivatives on TTX binding, but TEA and tetramethylammonium do not affect TTX binding (Reed and Raftery, 1976). Future electrophysiological studies of the interaction of guanidine derivatives with TTX at the sodium channel would yield useful information on the nature of the TTX binding site.

The effects of n-alkylguanidines on sodium conductance are very similar to those of quaternary derivatives of local anesthetics. We will develop a model based on Hille's (1977) concept of modulation of the local anesthetic receptor by channel gating. C$_8$ applied to the pronase-treated axon is the least complicated experimental system to model, since, in this case, the turning-on of sodium conductance appears to be normal, but, during maintained depolarization, C$_8$ causes the current to decay to a steady-state level, with a single exponential time-course. A reaction scheme which represents these processes is shown below.
Channel opening is controlled by the transition of resting (C) to open (O) channels. This transition is governed by the voltage-dependent rate constants $\alpha_m$ and $\beta_m$ of the Hodgkin-Huxley (1952) scheme (for our simulations we actually used the Moore-Cox (1976) model to give a closer fit of experimental currents). The transition of open to blocked channels (O$^*$) represents drug (Cn) binding to the channel and is governed by the voltage-independent rate constants $k$ and $l$. These constants can be determined directly from experimental data by the relationships $k = (1 - b)/\tau$ and $l = b/\tau$, where $b$ represents the steady-state fraction of unblocked channels, which can be determined from the ratio of current in the drug-treated axon to current in the control measured at the end of an 8-ms pulse. We measured $\tau$, the current decay time constant, at +90 mV because, at large depolarizations, $\alpha_m$ is very large, and the blocking reaction becomes the rate-limiting step that determines the time-course of sodium current decay. However, for voltage steps in the range of −20 to 0 mV, the channel closing rate constant, $\beta_m$, may limit the rate of entry of blocking molecules, thus causing an apparent increase in the time constant of sodium current decay.

Simulations of currents using this model can be compared with the observed sodium currents illustrated in Fig. 14. The values for $k$ and $l$ were calculated to be 0.7 and 0.4 ms$^{-1}$, respectively, on the basis of a steady-state block of 64% of the available channels and time constant of decay of 0.92 ms measured at +90 mV. At −10 mV, using the same $k$ and $l$, the decay time constant measured from simulated currents was 1.07 ms, compared with 1.03 ms from the observed currents. This model also correctly predicts the observed reduction of peak current at several Cn concentrations, as shown in Table III. At the molecular level, the blocking reaction may actually involve several steps, e.g., a slow hydrophobic binding step followed rapidly by channel occlusion by the guanidinium group. For short-chain derivatives, the hydrophobic step is not important, and channel block reaches equilibrium quickly with no apparent time dependence. This phenomenon can also be simulated in the model by increasing both $[C_n]$ and $l$.

Recovery from block occurs under resting conditions with a time constant of 200–300 ms. The simplest explanation is that the drug leaves the closed channel. The equilibrium for the reaction $C \leftrightarrow C^*$ would lie far toward $C$, such that at −70 to −100 mV the rate constant, $p$, would be negligibly small. Alternatively, as mentioned in Results, drug-blocked channels may not close, and removal of block is too slow to generate measurable tail current. With this addition, the model can simulate the cumulative effects of repetitive pulses.

In axons with normal sodium inactivation, the model predicts that after drug is applied, two processes operate to inactivate sodium channels, namely, the blocking reaction resulting from alkylguanidine molecules entering the channel and the natural inactivation reaction due to closing of the h gate. Thus, at all membrane potentials, the decay of sodium currents during depolarization should be accelerated. This effect was observed, as shown in Fig. 6. Similarly, decay of sodium current should be faster in the drug-treated h gate–intact axons compared with the pronase and drug-treated axons. Our
data conflict with this prediction, as shown by the comparison of the decay time constants of Figs. 6 and 8. Comparing the data from axons treated with 50 μM C₈ at depolarizations of +60 to +100 mV, the time constants from h gate-intact axons and pronased-treated axons were nearly the same. Furthermore, at depolarizations of -30 to 0 mV, decay time constants were greater in the h gate-intact axons. This discrepancy suggests that pronase treatment may facilitate drug binding to its receptor. Competitive interaction between

![Figure 14](image.png)

**Figure 14.** Comparison of C₈ block of sodium current in a pronase-treated axon (A) with block predicted by kinetic model (B). Same axon as in Fig. 7. Details of model in text.

**Table III**

| Drug Concentration (mM) | Axon Peak Steady-state | Model Peak Steady-state |
|------------------------|------------------------|------------------------|
| 0.02                   | 0.9                    | 0.4                    |
| 0.03                   | 0.7                    | 0.4                    |
| 0.20                   | 0.5                    | 0.4                    |

Bezanilla and Armstrong (1977) have shown that, theoretically, the voltage dependence of h can be explained by a physiological inactivation particle that blocks open channels with voltage-independent rate constants. Our experimental system, by providing a pharmacological model in which C₈ substitutes

guanidine derivatives and the h gate is compatible with the notion that the guanidino group of arginine is an important functional subunit of the h gate (Eaton et al., 1978).
for the inactivation particle in the pronase-treated axon, may provide a useful tool for investigating the molecular mechanism of sodium inactivation. However, there are several areas of dissimilarity between block by n-alkylguanidines and normal sodium inactivation. For instance, the voltage dependence of decay time-course is steeper for sodium inactivation (Fig. 6) than for n-alkylguanidine block (Fig. 8) in the potential range of -20 to +20 mV. However, this may be more of a quantitative than a qualitative discrepancy, because for both the model and the pronase-treated axon the time dependence of block becomes very sensitive to voltage in the range of -30 to -20 mV. In fact, at -40 mV, sodium current does not appear to decay during an 8-ms pulse in the model. A more serious discrepancy arises from the difference between the time-course of recovery from sodium inactivation (time constant, 4 ms at $E_m = -80$ mV) and that of recovery from n-alkylguanidine block (time constant, 210 ms at $E_m = -80$ mV). This difference probably reflects a real departure of the drug-induced inactivation mechanism from that of sodium inactivation. Another important dissimilarity, which has been observed by others, is that, whereas TTX binding is inhibited by n-alkylguanidine (Reed and Trzos, 1979), it is not altered by depolarization that would inactivate sodium channels (Almers and Levinson, 1975).

Although there are clear differences between the molecular mechanisms of n-alkylguanidine block and sodium inactivation, n-alkylguanidine block is a useful demonstration of how the inactivation and activation gates, although pharmacologically separable by pronase treatment, can be kinetically coupled.

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