# LOCAL ANESTHETIC BLOCK OF SODIUM CHANNELS IN NORMAL AND PRONASE-TREATED SQUID GIANT AXONS

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ABSTRACT The inhibition of sodium currents by local anesthetics and other blocking compounds was studied in perfused, voltage-clamped segments of squid giant axon. When applied internally, each of the eight compounds studied results in accumulating "use-dependent" block of sodium currents upon repetitive pulsing. Recovery from block occurs over a time scale of many seconds. In axons treated with pronase to completely eliminate sodium inactivation, six of the compounds induce a time- and voltage-dependent decline of sodium currents after activation during a maintained depolarization. Four of the time-dependent blocking compounds—procaine, 9-aminoacridine, *N*-methylstrychnine, and QX572—also induce altered sodium tail currents by hindering closure of the activation gating mechanism. Treatment of the axon with pronase abolishes use-dependent block completely by QX222, QX314, 9-aminoacridine, and *N*-methylstrychnine, but only partially by tetracaine and etidocaine. Two pulse experiments reveal that recovery from block by 9-aminoacridine or *N*-methylstrychnine is greatly accelerated after pronase treatment. Pronase treatment abolishes both use-dependent and voltage-dependent block by QX222 and QX314. These results provide support for a direct role of the inactivation gating mechanism in producing the long-lasting use-dependent inhibition brought about by local anesthetic compounds.

## INTRODUCTION

Local anesthetics block the sodium channels of nerve membranes by a different mechanism from that of tetrodotoxin (TTX) or saxitoxin (STX). Although TTX and STX are active only at the outer surface of the membrane (Koppenhöfer and Vogel, 1969; Narahashi, 1971), neutral and amine anesthetic molecules are active when applied either inside or outside the cell, and most permanently charged anesthetic derivatives are active only from the inside (Frazier et al., 1970). It seems likely from several lines of evidence that externally applied neutral and amine anesthetics reach their blocking sites by diffusing into and through the membrane (see Hille, 1977*a*). In addition, in contrast to TTX or STX, the degree of blocking by amine anesthetics and their charged derivatives depends upon membrane potential and the previous pulse history. Block accumulates at a pulse frequency of 1 Hz and even lower stimulation rates, because recovery from block proceeds slowly and is incomplete during the interpulse interval. This type of blocking behavior by local anesthetic compounds has been previously

described for the frog node of Ranvier (Strichartz, 1973; Courtney, 1975; Khodorov et al., 1976; Hille, 1977*a*, *b*) and frog skeletal muscle (Schwarz et al., 1977), and is described in this paper for the squid giant axon. The term "use-dependent" or "frequency-dependent" block has been applied because the degree of block depends upon how often sodium channels are opened. This action can limit the frequency of firing to rates lower than normal, resulting in an antiarrhythmic type of pharmacological effect.

Normally there are two factors or processes controlling the openness of sodium channels: the rapid activation or opening of channels upon depolarization and the slower, subsequent inactivation or closing of channels as the depolarization is maintained. These two gating processes, termed $m^3$ and $h$, respectively, by Hodgkin and Huxley (1952), result in a transient sodium current during a maintained depolarization, making the kinetics of the interaction between anesthetic molecules and open channels more difficult to study. One motivation in the experiments reported here was to investigate the kinetics of block by a variety of compounds in open channels. Using pronase, a mixture of proteolytic enzymes, perfused inside a squid giant axon, the normal sodium inactivation process can be completely eliminated (Armstrong et al., 1973). After pronase treatment, channels remain open for seconds during a maintained depolarization. Then, after perfusion with the test anesthetic compound, the rate of anesthetic block may be directly measured as a time-dependent decline of the sodium current during a single depolarizing pulse. Many of the anesthetic compounds act as artificial channel gates that close the channel after it has opened. Pronase has been used to simplify the kinetic interaction between drug and channel by Yeh and Narahashi (1977) to study block by pancuronium and by Rojas and Rudy (1976) to study block by tetraethylammonium derivatives. In addition, *Leirus* scorpion venom (Shapiro, 1977) and batrachotoxin (Khodorov, 1978) have been used to modify sodium inactivation, allowing time-dependent blocking of sodium channels to be visualized.

The interaction between the sodium channel gating mechanism and local anesthetic compounds has been described in a kinetic hypothesis (Courtney, 1975; Hille, 1977*b*), termed the "modulated receptor hypothesis" by Hille. In this hypothesis, the sodium inactivation gating mechanism plays a key role in bringing about long-lasting use-dependent inhibition. Again using pronase, it is possible to test the idea that use-dependent inhibition directly involves the inactivation mechanism. All of the compounds studied in this investigation induce use-dependent block in axons with normal sodium channel gating, and pronase treatment abolishes use dependence for several, though not all, of the compounds. The elimination of use-dependent block by pronase treatment has been briefly reported for strychnine (Cahalan and Shapiro, 1976), 9-aminoacridine (Yeh and Narahashi, 1976), and QX314 (Almers Cahalan, 1977).

## MATERIALS AND METHODS

Segments of giant axons averaging 420 $\mu$m in diameter from *Loligo pealii* were fine-cleaned, internally perfused, and voltage-clamped as described by Cahalan and Begenisich (1976). For

TABLE I

| Solutions | [Na] | [Ca] | [Mg] | Tris | Cl | pH |
|---|---|---|---|---|---|---|
| | | | *mM* | | | |
| External | | | | | | |
| Na ASW* | 440 | 10 | 50 | 10 | 570 | 7.4 |
| Tris ASW | — | 10 | 50 | 475 | 565 | 7.4 |

| | [Na] | [K] | [TEA]‡ | Glutamate | F | $H_2PO_4$ | Sucrose | pH |
|---|---|---|---|---|---|---|---|---|
| Internal | | | | *mM* | | | | |
| SIS§TEA | — | 375 | 25 | 320 | 50 | 15 | 300 | 7.2 |
| 50Na SIS TEA | 50 | 325 | 25 | 320 | 50 | 15 | 300 | 7.2 |
| 200 Na | 200 | — | — | 150 | 50 | — | 510 | 7.2 |

*Artificial sea water.
‡Tetraethylammonium.
§Standard internal solution.

96 axons, the average resting potential was −60 mV when internally perfused by a standard internal solution (see Yeh and Narahashi, 1977, and Table I), and bathed in K-free artificial sea water. Papain (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml) rather than pronase was used as a milder enzyme treatment to initiate the perfusion. Usually just the end of the axon outside the current-measuring region was exposed to the enzyme solution for 1–2 min to minimize possible effects of the enzyme solution on sodium inactivation. During the experiment, both internal and external solutions were continuously exchanged by gravity flow at 3–6 vol/min. The temperature was monitored by a thermistor next to the axon and controlled by a Peltier device (Cambion, Cambridge, Mass.) at 12°C in most experiments. Table I indicates the external and internal control solutions. Internal solutions with potassium contained 25 mM tetraethylammonium (TEA) to block ionic currents through potassium channels. Sodium solutions were also used internally to increase the outward current magnitudes, and as a control for possible interference of the anesthetic blocking action by TEA ions in the solutions containing potassium. Internal and external solutions were isosmotic within 2% of 1,010 mosmol/kg.

In experiments with altered sodium inactivation, the axon was pretreated internally with 1 mg/ml pronase (Pronase CB, Calbiochem, San Diego, Calif.) for 5–10 min at 12°C as the sodium currents were monitored for effects on sodium inactivation. When inactivation removal was nearly complete, the pronase was washed out. Treating the ends of the axon outside the chamber with 0.5 μm TTX prevented long-duration active responses in regions out of voltage-clamp control. After pronase treatment, axon run-down was normally accelerated. In a few experiments, inactivation was removed with 0.5 mM *N*-bromoacetamide, kindly provided by Dr. G. Oxford (see Oxford et al., 1976).

Fig. 1 illustrates the compounds used in these experiments. The clinically used tertiary amine anesthetics, tetracaine and etidocaine, as well as the quaternary lidocaine derivatives, QX222, QX314, and QX572, were kindly provided by Dr. Bertil Takman of the Astra Pharmaceutical Products, Inc., Worcester, Mass. Procaine and 9-aminoacridine were purchased from Sigma Chemical Company, and *N*-methylstrychnine was a generous gift from Dr. B. Shapiro, who participated in some of the early experiments (see Cahalan and Shapiro, 1976). The effects of these eight compounds were studied on the axoplasmic side of the membrane in a concentration range from 80 μM to 2 mM. As pointed out by Hille (1977*a*), lipid-soluble compounds that cross the membrane rapidly could well be less concentrated in unstirred layers next to the membrane than in the perfusion solution. In fact, in experiments with internally perfused

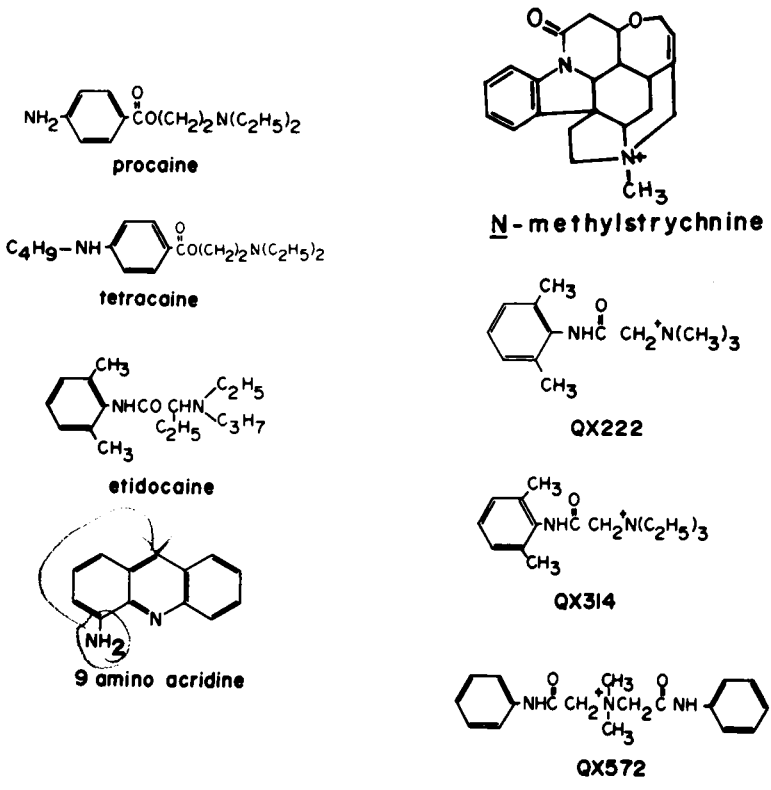



FIGURE 1 Chemical structures of blocking compounds. The left side shows tertiary amine compounds. The right side illustrates cationic derivatives of strychnine (top) and lidocaine.

9-aminoacridine or strychnine (but not *N*-methylstrychnine), when the perfusion was deliberately stopped, the blocking effects of these compounds on the sodium conductance gradually disappeared in a couple of minutes. On restarting the perfusion, the effects immediately reappeared. This escape of the blocking molecules across the membrane to the external solution was counteracted either by using quaternary derivatives such as *N*-methylstrychnine or by placing an equal concentration of the tertiary compound on both sides of the membrane. Even so, the unstirred layer effects and partitioning into membranes result in some uncertainty as to the concentration of blocking molecules near the axoplasmic surface of the membrane.

The membrane potential, $E_m$, defined as the inside minus the outside potential, was corrected for liquid junction potentials at both the inner pipette filled with 560 mM KCl and the external sea water bridge. The holding potential in these experiments was −70 mV. Compensation for a series resistance of 3Ω-cm$^2$ was employed throughout.

The total membrane current was displayed on an oscilloscope and photographed directly or digitized and stored temporarily on shift register buffers of a signal averager (Bezanilla and Armstrong, 1977). By using the signal averager with both analog and digital subtraction of the linear portion of ionic and capacity current, a time resolution for current measurement of 10 $\mu$s was achieved. The contents of the shift register buffer could be stored on cassette tape and later transferred to a PDP 8 computer for analysis (Digital Equipment Corp., Maynard, Mass.).

## RESULTS

This paper investigates the interaction of eight anesthetic and related compounds with the sodium channel activation and inactivation gating mechanisms. In axons with normal gating, each compound studied results in use-dependent inhibition of the sodium current, similar to that observed in frog myelinated nerve and skeletal muscle fibers. The kinetics and voltage dependence of block by anesthetic compounds in axons pretreated with pronase are presented in the second part of this section. The following part then describes recovery from block and the elimination of use dependence in pronase-treated axons. Finally, the effects of anesthetic compounds on sodium tail currents are described.

### *Use-Dependent Block in Axons with Normal Sodium Inactivation*

After adding one of the blocking agents inside the axon, two types of sodium current block are produced: the so-called "tonic" inhibition (Strichartz, 1973), a decline in the sodium current without conditioning pulses, and use-dependent modulation of sodium current by conditioning voltage-clamp pulses. At the holding potential of −70 mV used in these experiments, tonic inhibition sometimes amounted to 50%, but never as great as the 90% tonic block observed by Strichartz (1973) with QX314. An example of use-dependent modulation of the sodium current is shown in Fig. 2 A for

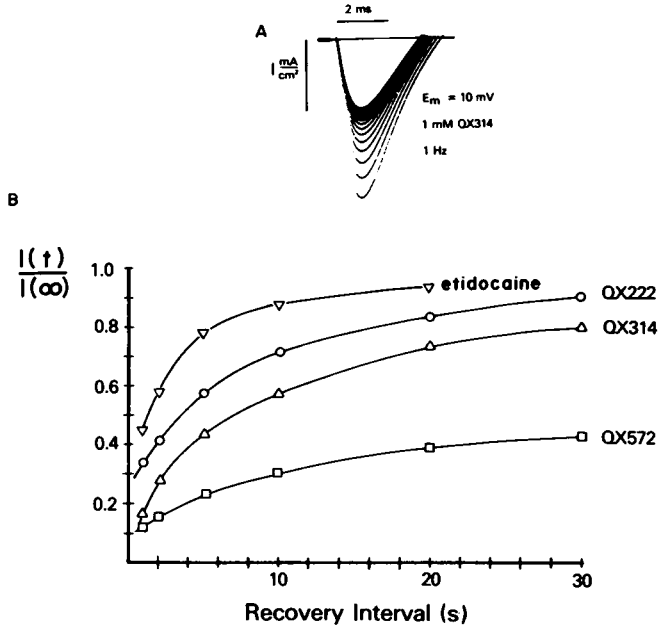



FIGURE 2 (A) Use-dependent block of sodium channels by 1 mM QX314 applied inside. Sodium currents under voltage clamp are shown for 15 pulses, each lasting 6 ms, to a membrane potential of 10 mV every second. (B) Recovery from use-dependent block at −70 mV for four compounds. Solutions: Na artificial seawater (ASW)//Standard internal solution (SIS) tetraethylammonium (TEA).

the compound QX314. After a long rest period in the presence of 1 mM QX314 applied internally, successive voltage-clamp pulses to +10 mV produce smaller and smaller sodium currents until a steady level of inhibition is attained. The inhibition is greater at higher pulse frequencies and for stronger depolarizations.

Local anesthetic block accumulates at a frequency of 1 Hz and even lower stimulation rates, because recovery from block occurs over a time scale of many seconds or even minutes, as shown in Fig. 2 B for four of the compounds studied. To obtain these recovery curves in four separate experiments, the axon was conditioned with 10 voltage-clamp pulses to +110 mV to achieve maximum inhibition of the sodium current, and then tested with a single pulse to +10 mV at variable times to follow the recovery from use-dependent block. The recovery rate varies for different compounds, as summarized in Table II. This slow recovery from block at the holding potential accounts for the progressive decline of sodium currents during repetitive pulsing, provided that recovery from block is incomplete during the interpulse interval. In many ways, the process resembles slow inactivation of the sodium conductance that normally is present in *Myxicola* axons (Rudy, 1975, 1977).

Fig. 3 illustrates use-dependent block brought about by 9-aminoacridine (9AA) as a qualitative illustration of two properties brought about by each of the compounds. The top two sets of records demonstrate that use-dependent block is stronger at more positive depolarizing voltage pulses. The modulation of sodium current magnitude with repetitive pulsing occurs over a wider range for pulses to 110 mV on the top right than for pulses to 10 mV on the top left. The voltage dependence of anesthetic block will be described in greater detail later in this section.

The bottom records in Fig. 3 indicate that inactivated channels are less susceptible to use-dependent block. In these records, a control pulse to 10 mV activates a large inward sodium current of about 2 mA/cm$^2$. Then 10 conditioning pulses to 110 mV result in use-dependent block of most of the channels. On the left-hand set of records, the outward currents can be seen to decline on each successive pulse to 110 mV. A test pulse to 10 mV after conditioning produces much less sodium current (0.5 mA/cm$^2$)

TABLE II

CHARACTERISTICS OF USE-DEPENDENT BLOCK IN NORMAL AXONS

| Compound | Concentration | $T_{1/2}$ recovery | Steepness of voltage dependence $\delta$ | Modulation |
|---|---|---|---|---|
| | *mM* | *s* | | % |
| Etidocaine | 0.25–1 | 3.0 ± 0.5 | 0.83 ± 0.04 | 0–70 |
| Tetracaine | 0.5–1 | 3.5 ± 0.5 | 0.95 | 0–50 |
| NMS | 0.5–1 | 6 | 0.90 | 0–60 |
| 9AA | 0.17 | 6 | 0.88 | 0–70 |
| QX222 | 1 | 7.75 ± 0.25 | 0.89 | 0–60 |
| QX314 | 1 | 14.5 ± 0.5 | 0.86 ± 0.04 | 0–85 |
| QX572 | 0.25–1 | 50 | — | — |

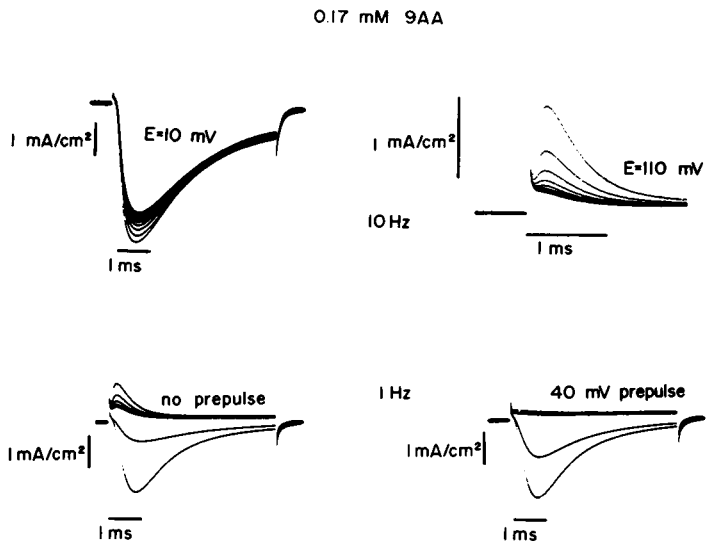



FIGURE 3 Use-dependent block by 9AA. Top left: repetitive pulses to 10 mV at 10 Hz. Top right: repetitive pulses to 110 mV at 10 Hz. The lower traces illustrate the control, conditioning, test-pulse protocol. At 1 Hz, first a control pulse is given to 10 mV, followed by 10 conditioning pulses to 110 mV (outward sodium currents), and finally a test pulse to 10 mV. In the bottom right traces a prepulse to −30 mV for 50 ms preceded each conditioning pulse to inactivate the outward sodium current. Solutions: Na ASW//SIS TEA.

than the original control pulse. The ratio of the current during the test pulse compared with the control pulse provides a measure of the block produced during the conditioning pulses. This is a reasonable measure because recovery from use-dependent block is so slow, proceeding with a half-time of 6 s in the case of 9AA. In the right-hand traces, a 40-mV depolarizing prepulse of 50-ms duration immediately preceded each conditioning pulse, inactivating the outward sodium currents. The test pulse to 10 mV then produces about 1 mA/cm$^2$ sodium current after the conditioning pulses, roughly twice that in the left-hand set of traces, even though the axon was depolarized both by the conditioning pulses to 110 mV and by the 40-mv prepulses. It appears, then, that inactivated channels interact less readily with anesthetic molecules. This result is also in qualitative agreement with the features of use-dependent block in myelinated nerve fibers (Strichartz, 1973).

To obtain a more quantitative measure for the voltage dependence of block, the control, conditioning, test pulse protocol was extended to variable conditioning potentials. Fig. 4 illustrates this protocol before and after 1 mM etidocaine was added to the internal perfusion solution. Before the addition of etidocaine, the currents during the control and test pulse superimpose, and no use-dependent block is observed during the 10 conditioning pulses. After etidocaine is added, there is progressive block during the conditioning pulses, and the current during the test pulse is substantially reduced compared with the current during the control pulse. In Fig. 4 B the ratio of

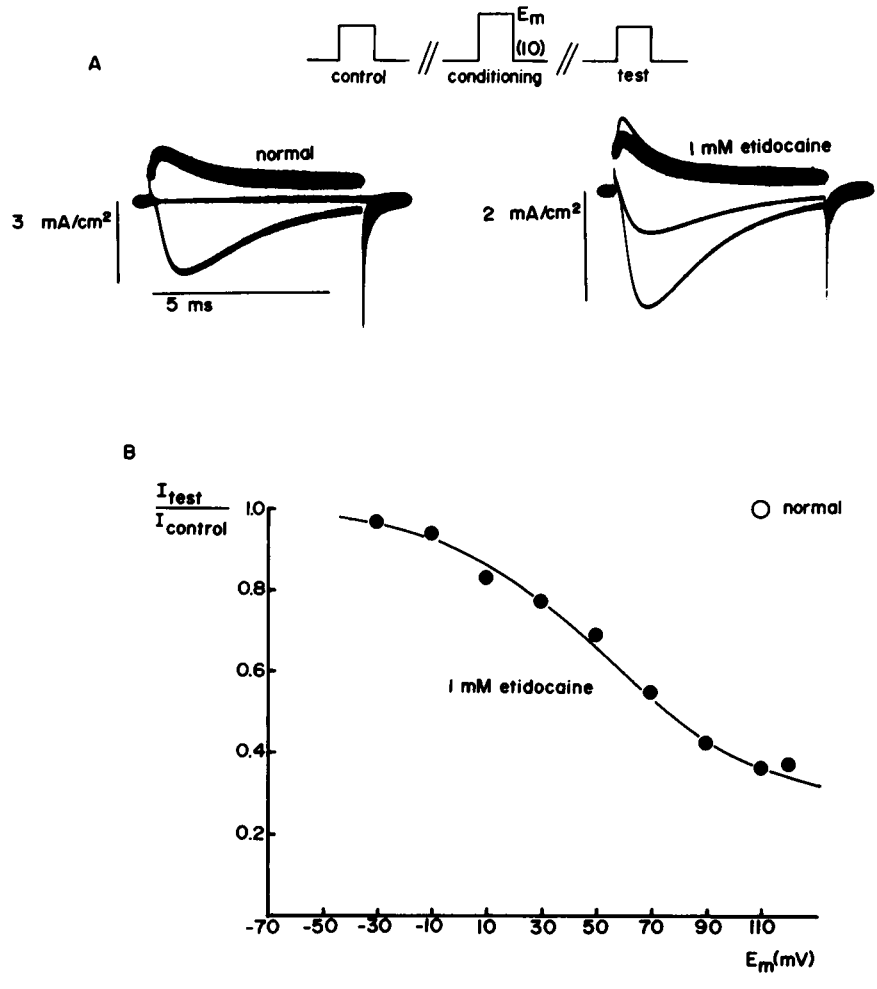



FIGURE 4 Control (10 mV), conditioning ($E_m$), and test pulse (10 mV) protocol for a normal axon without blocking compound on the top left, and after adding 1 mM etidocaine internally on the top right. The pulse pattern is diagramed above. The graph below plots current during the test pulse ($I_{test}$) divided by current during the control pulse ($I_{control}$) as a function of the conditioning pulse potential ($E_m$). The smooth curve fitted to the data points for 1 mM etidocaine represents Eq. 1 with $B = 0.28$ and $\delta = 0.85$. Solutions: Na ASW//SIS TEA.

test current over control current is plotted against conditioning pulse potential. As the conditioning potential is made more positive, use-dependent block proceeds to a deeper steady-state level.

The smooth curve fitted to the data points in Fig. 4 B represents the equation:

$$I_{test}/I_{control} = \frac{1 - B}{1 + \exp\left[(E - \bar{E})/s\right]} + B,$$

where $B$ is a base line of maximal use-dependent block; $E$ is the membrane potential;

$\bar{E}$ is the potential at the inflection point of the curve, and $s$ is an adjustable parameter that determines the maximum slope of the curve. The equation is normalized between a base line of maximal inhibition, $B$, and zero inhibition. (Another example of this type of fitting is illustrated in Fig. 10 B.) The parameter $s$ can also be written as $RT/z\delta F$, where $F$, $R$, and $T$ are the Faraday and the gas constant, and the absolute temperature, respectively, and $z\delta$ is the charge times a fraction of the electric field across the membrane. The parameter $\delta$ has been taken to represent an equivalent electrical distance across the membrane from inside to outside (see Woodhull, 1973; Strichartz, 1973). The voltage dependence of use-dependent block might reflect the distance across the membrane electric field that a cationic blocking molecule would have to travel to reach its receptor inside the channel. Alternatively or in addition, the formation of a receptor for local anesthetic block might be voltage dependent. Other possible mechanisms could alter the steepness of voltage-dependent block, including effects of ionic current and/or gating on the level of block. Thus, the parameter $\delta$ should not be taken literally to indicate the location of a site within the channel, but it does serve as a quantitative index in comparing different blocking agents. The steepness parameter, $\delta$, was determined for six of the anesthetic compounds tested by fitting the above equation to the data from conditioning pulse experiments. In each case, the steepness of the voltage dependence suggests transfer of one charge from 80 to 95% of the way across the membrane field during the blocking reaction.

The principal features of use-dependent block by seven compounds in axons with unaltered sodium channel gating are summarized in Table II. Sodium currents recover slowly from block by each compound with a time-course ranging from 3 to 50 s. This time-course determines at which frequencies accumulating block will occur with repetitive pulsing. The degree of use-dependent modulation with repetitive pulses at 1 Hz varies from 50 to 85%, and the steepness parameter for voltage-dependent blocking falls within a range from 0.79 to 0.95. In general, the effect of each compound on the sodium current is quite similar, and the properties of block are like those in frog myelinated nerve and muscle fibers. The next section will illustrate how pronase treatment reveals differences between the compounds regarding their interaction with the channel gating mechanism.

*Anesthetic Block in Pronase-Treated Axons*

Recent hypotheses for local anesthetic action account for use-dependent block in terms of interactions with a receptor inside the sodium channel (Strichartz, 1973; Courtney, 1975; Hille, 1977*a*, *b*; Schwarz et al., 1977). Access to the receptor by protonated amines or quaternary ammonium compounds in the axoplasm is restricted by the gating mechanism; the gates must open before blocking molecules can interact with the receptor. The interaction blocks the open channel, and slow voltage-dependent binding to the inactivation mechanism results in long-lasting inhibition, according to the hypothesis. After pronase treatment, it is possible to observe the anesthetic interaction with open channels more directly.

After adding 1 mg/ml pronase to the internal perfusion solution of a squid axon,

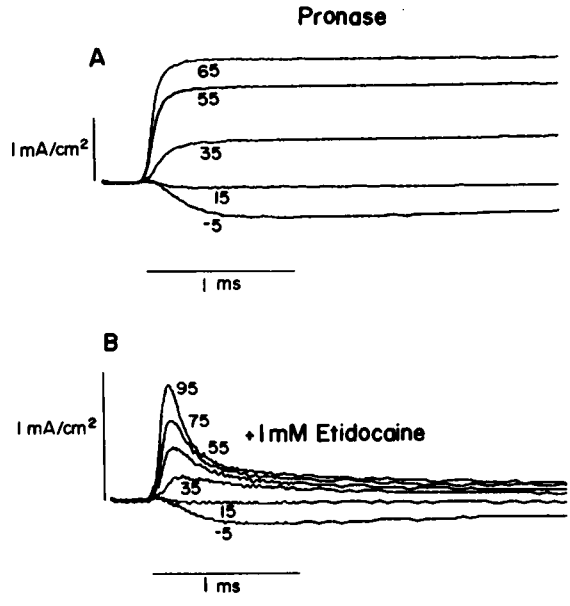



FIGURE 5 (A) Sodium currents after pronase treatment. The membrane potential is indicated next to each current trace. (B) Sodium currents after adding 1 mM etidocaine internally. Solutions: Na ASW//200 Na.

the sodium inactivation process begins to fail and eventually can be completely destroyed, as illustrated in Fig. 5 A. The effects of pronase are irreversible, and it is necessary to wash out the enzyme to avoid axon deterioration. With complete pronase treatment, channels open upon depolarization and remain open for seconds, although ultraslow sodium inactivation still persists (Rudy, 1977). After pronase treatment, several anesthetic compounds applied internally induce a change in the sodium current time-course. An example of this effect is illustrated in Fig. 5 B after perfusion with 1 mM etidocaine. The sodium currents activate, reach a peak, and then decline. Inward currents for small depolarization and outward currents for larger depolarization appear to "inactivate" in the presence of etidocaine and several other blocking agents. This effect resembles the action of internal TEA derivatives on potassium channels (Armstrong, 1969).

Fig. 6 illustrates sodium current block induced by 9AA, *N*-methylstrychnine, etidocaine, and QX222 in pronase-treated axons. In parts A, B, and D control traces before adding the blocking compounds are included. Each compound produces block, though with QX222 and QX314 (not illustrated) the currents appear to be simply scaled relative to the control, in contrast to the time-dependent block brought about by the other compounds. Comparing Fig. 6 C with Fig. 5 B illustrates that block by etidocaine is quicker and more complete at higher drug concentrations. These records show that open channels do become blocked by compounds that induce use-dependent block when inactivation gating ($h$) is intact. If inactivation is removed by treatment with *N*-bromoacetamide (Oxford et al., 1976) rather than pronase, a similar time-

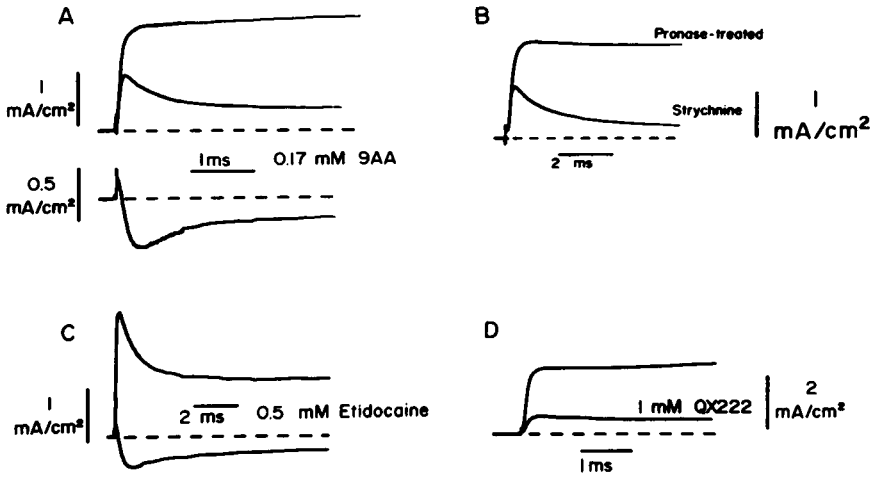
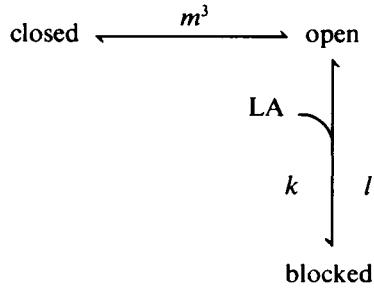



FIGURE 6 Block of $I_{Na}$ by four compounds in pronase-treated axons. Control traces are included in parts A, B, and D after pronase treatment but before adding the blocking compound. (A) Top two traces for pulse to +90 mV before and after adding 0.17 mM 9AA. Bottom trace shows inward current after adding 9AA for pulse to 10 mV. (B) Current during pulse to +90 mV before and after adding 1 mM NMS inside. (C) Current after adding 1 mM etidocaine inside for pulses to 10 and 90 mV. (D) Current before and after adding 1 mM QX222 for pulse to 90 mV. Solutions: Na ASW//50 Na SIS TEA.

dependent block occurs (not illustrated). In addition, Shapiro (1977) demonstrated the same type of effect, using strychnine to block channels with inactivation gating altered by *Leiurus* scorpion venom, and Khodorov (1978) showed block induced by trimecaine in channels with activation and inactivation gating modified by batrachotoxin. Thus, the interaction with open channels becomes apparent with a variety of chemical alterations of the inactivation process.

A simple possible kinetic model for anesthetic block after pronase treatment postulates that the sodium channel opens normally by Hodgkin-Huxley $m^3$ kinetics upon depolarization and then becomes blocked by local anesthetic (LA):




The transition from closed to open can be represented by four states: $v \underset{\beta}{\overset{3\alpha}{\rightleftarrows}} w \underset{2\beta}{\overset{2\alpha}{\rightleftarrows}} x \underset{3\beta}{\overset{\alpha}{\rightleftarrows}}$ open, with $\alpha$ and $\beta$ representing the Hodgkin-Huxley rate constants for the

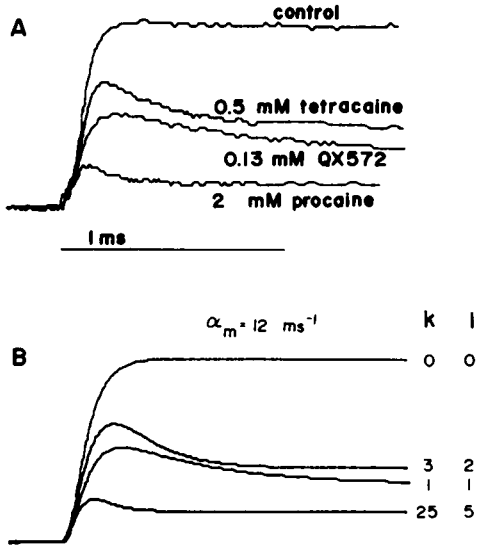



FIGURE 7 (A) Block of $I_{Na}$ at 90 mV by tetracaine, QX572, and procaine in pronase-treated axons. Sodium current was scaled to a control trace just before adding blocking compound. Solutions: Na ASW//50 Na SIS TEA. (B) Kinetic simulation of currents in part A with blocking and unblocking rates, $k$ and $l$, indicated for each trace. The channel opening rate, $\alpha_m$, = 12 ms$^{-1}$ in each trace.

activation of sodium conductance. The rate constants $k$ for blocking the channel and $l$ for leaving the channel determine the time-course and steady-state level for the decline of the sodium current. The model is patterned after Armstrong's (1969) kinetic description of potassium channel block by TEA derivatives.

Fig. 7 illustrates fits of the model to the time-course of sodium currents before and after addition of three blocking compounds, in three separate experiments. In each case, the control trace before adding the blocking compound for a pulse to +90 mV has been scaled to superimpose. The time-course of the control trace was fitted with $\alpha_m$ = 12 ms$^{-1}$ and $\beta_m$ = 0. To fit the time-course of the sodium current after adding the blocking compound, $k$ and $l$ were varied empirically until there was close agreement of the time-course. In the case of QX572, it was also necessary to scale the current by 0.62. This factor, which represents a 38% "tonic block," was not required for the other compounds in this figure. As $k$ is made larger, the currents peak more rapidly and the steady-state level of block, $k/k + l$, is increased. Procaine blocks and unblocks more rapidly than tetracaine or QX572. Larger, more hydrophobic compounds such as QX572, tetracaine, etidocaine, 9AA, and strychnine have blocking rates slow in comparison to the rate of channel opening. With QX222 (see Fig. 6 B and Fig. 9) and QX314 (not illustrated), the currents seem to be simply scaled in amplitude with respect to the control. These relatively hydrophilic compounds might equilibrate too rapidly with open channels to produce a change in the sodium current time-course; if $k$ and $l$ are large compared to $\alpha_m$, little or no change in time-course is expected.

Alternatively, pronase treatment may alter the normal blocking interaction of these relatively hydrophilic compounds. These two possibilities will be considered later in terms of the voltage dependence of block induced by QX222.

The simple model presented above does not in every case accurately fit the time-course of sodium currents after addition of blocking compound because of a slow component of block that may be observed in Fig. 5 B for 1 mM etidocaine. After an initial rapid phase of decline, there is a slower component not predicted by the closed $\rightleftarrows$ open $\rightleftarrows$ blocked model. This more complex time-dependent blocking was consistently observed with etidocaine, strychnine, or *N*-methylstrychnine, and tetracaine. Therefore, the simple fitting procedure illustrated in Fig. 7 could not be applied to analyze the voltage dependence of block by these compounds. However, it is apparent that for stronger depolarizations, the time-course of block is faster. With 9AA, the time-course of block can be fitted by the simple kinetic model presented above, with a single exponential decline for "inactivation" induced by 9AA. Table III presents $k$ and $l$ values at various membrane potentials with 80 $\mu$m 9AA inside the axon. The fitting procedure was first to estimate $\alpha_m$ for control traces before adding 9AA at varying membrane potentials. The traces after adding 9AA were then fitted by using the appropriate $\alpha_m$ value at each membrane potential and adjusting $k$ and $l$ to match the time-course of block. As Yeh and Narahashi (1977) have shown for block by pancuronium ions, the 9AA entry rate, $k$, is nearly voltage independent, whereas the exit rate, $l$, has a definite voltage dependence with slower exit rates at higher potentials. The maximum steepness of this voltage dependence corresponds to an $e$-fold decrease in $l$ per 28 mV depolarization.

Another way to examine the voltage dependence of block is to measure the fraction of channels blocked in the steady state at varying membrane potentials. In axons treated with pronase to eliminate sodium inactivation completely, this is done by comparing the current at the end of a long-duration voltage-clamp pulse before and after adding the blocking compound. The blocking compound induces a decline in the sodium current to a steady level that depends upon membrane potential. The steady-state voltage dependence for 9AA and etidocaine in pronase-treated axons is illustrated in Fig. 8. In Fig. 8 A the axon was perfused with 200 mM Na and bathed in Na-free Tris sea water or normal Na sea water. Voltage-clamp currents were recorded

TABLE III
PARAMETERS FOR 9AA BLOCK IN PRONASE-TREATED AXON

| $E$ | $\alpha$ | $k$ | $l$ |
|---|---|---|---|
| $mV$ | $ms^{-1}$ | $ms^{-1}$ | $ms^{-1}$ |
| +10 | 6.5 | 1.3 | 0.8 |
| +30 | 8.5 | 1.3 | 0.4 |
| +50 | 12 | 1.3 | 0.2 |
| +70 | 13 | 1.3 | 0.2 |
| +90 | 14 | 1.4 | 0.15 |

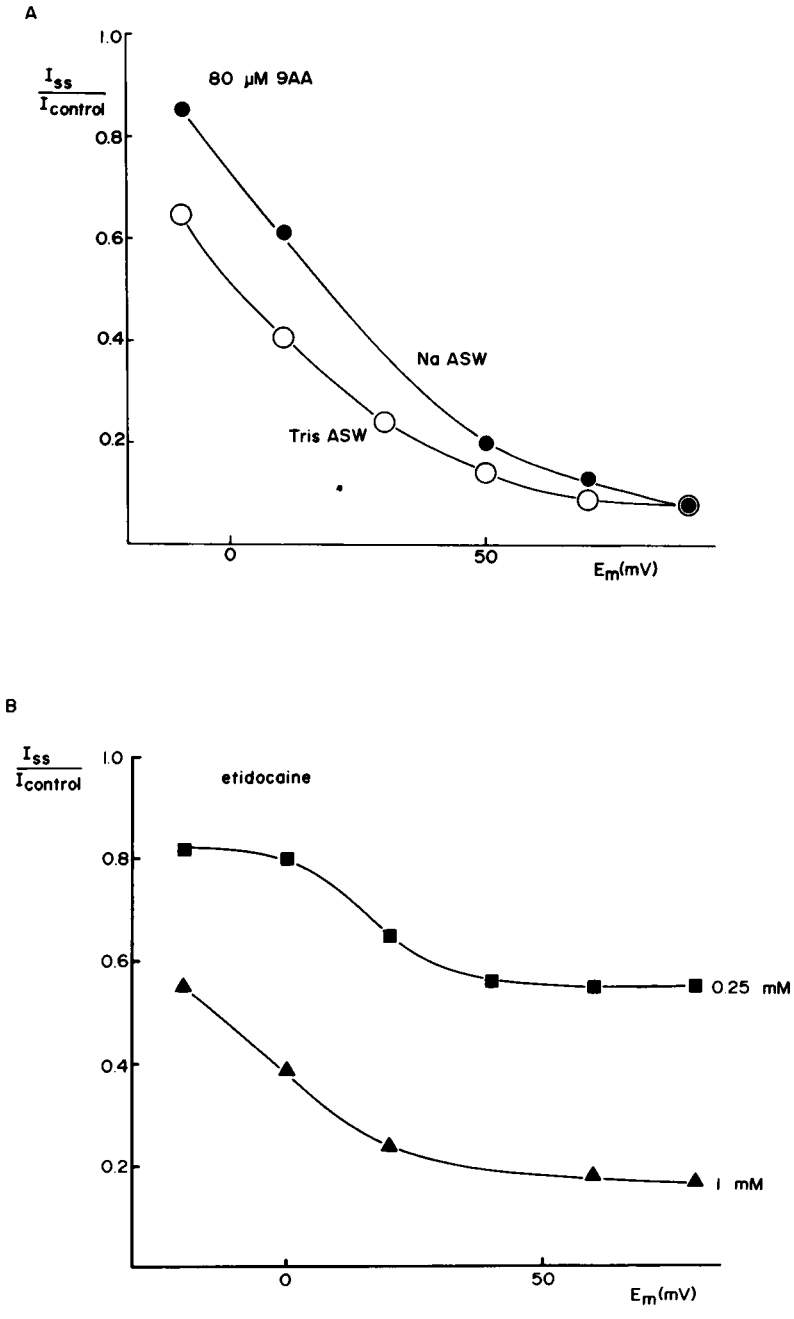



FIGURE 8 Steady-state voltage dependence for block in a single pulse by 9AA (A) and etidocaine (B). The ratio of sodium currents at the end of an 8-ms depolarization before ($I$ control) and after ($I_{ss}$) adding blocking compound is plotted against each membrane potential ($E_m$). (A) Solutions: Na ASW (•) and Tris ASW (○)//200 Na. (B) Solutions: Na ASW//50 Na SIS TEA.

for potentials from −10 mV to +90 mV before and after adding 80 μM 9AA to the inside of the axon. In the steady state, more block was produced by 9AA for stronger depolarizing pulses. In the presence of external sodium, some of the block was relieved at less positive potentials, resulting in a slight steepening of the voltage dependence. Current-dependent block of sodium channels has been reported for strychnine (Shapiro, 1977; Cahalan and Shapiro, 1976), and should be examined further for other blocking compounds. Like 9AA, etidocaine blocks a greater proportion of channels at more positive membrane potentials. Fig. 8 B illustrates block at two concentrations of etidocaine. Higher concentrations and more positive potentials augment the inhibition.

After pronase treatment, QX222 and QX314, in contrast to the six other compounds studied, do not alter the time-course of the sodium conductance greatly, if at all. Do QX222 and QX314 simply result in tonic block after pronase treatment, or are the rate constants for the blocking interaction, $k$ and $l$, much larger than for opening the channel? If these relatively hydrophilic molecules do equilibrate rapidly in a voltage-dependent interaction with open sodium channels, there should be a change in the shape of the sodium current-voltage relationship. An example of rapid voltage-dependent block is the effect of internal sodium ions on potassium channels (Bezanilla and Armstrong, 1972; French and Wells, 1977). Sodium ions block potassium channels at depolarized potentials and induce negative resistance in the potassium current-voltage relation. Fig. 9 illustrates that the shape of the sodium current-voltage relations is hardly affected by QX222. Nearly the same amount of block occurs at each potential; the average block is 42%. In axons with normal inactivation, both QX222 and QX314 induce voltage-dependent block established with repetitive pulsing. Thus, pronase treatment reduces or abolishes the voltage dependence of block established by QX222 and QX314 with single pulses.

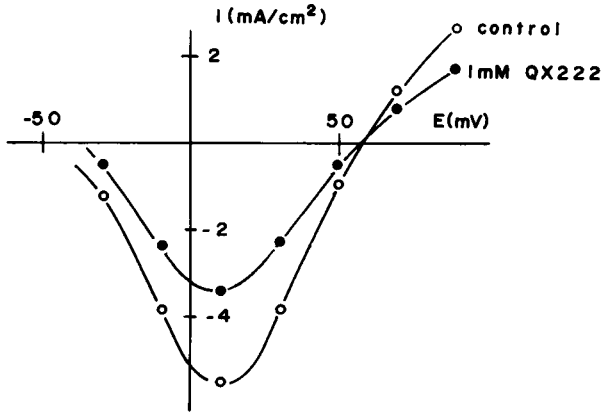



FIGURE 9 Steady-state sodium current-voltage relation in pronase-treated axon before and after internal addition of 1 mM QX222. Curves drawn by eye.

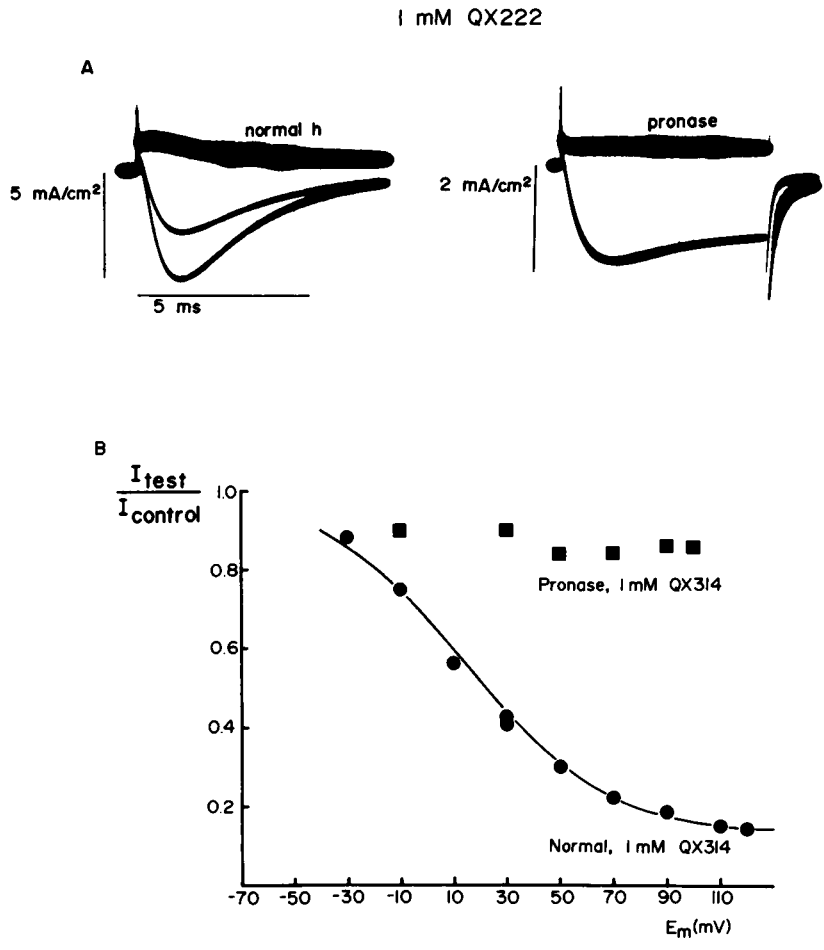



FIGURE 10 Elimination of use-dependent block by pronase treatment. (A) Traces on left illustrate sodium currents for a control pulse to +10 mV, 10 conditioning pulses to +190 mV, and a test pulse to +10 mV with 1 mM QX222 inside the axon before pronase treatment. After pronase treatment (right traces), the same pulse protocol was repeated again with 1 mM QX222 present. Solutions: Na ASW//SIS TEA. (B) Voltage dependence of QX314 block determined by conditioning pulse protocol. In normal axon, large depolarizing pulses lead to greater use-dependent block by QX314. In pronase-treated axon, the conditioning pulse potential results in very little inhibition of sodium current during the pulse compared with the control pulse. Solutions: Na ASW//SIS TEA.

### *Recovery from Block in Pronase-Treated Axons*

Experiments in pronase-treated axons provide a test for the role of inactivation gating in use-dependent block. Each of the compounds studied results in use-dependent inhibition in axons with normal inactivation gating. Pronase treatment almost completely abolishes the long-lasting use-dependent nature of anesthetic block for QX222,

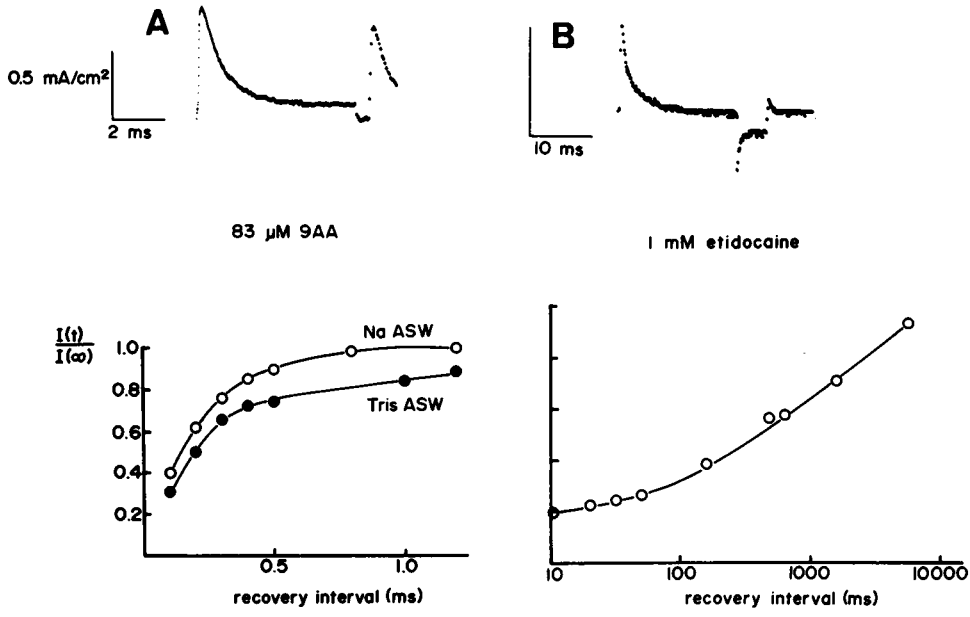



FIGURE 11 Recovery from block by 9AA (A) and etidocaine (B) in two pulse experiments after pronase treatment. Two pulses to +90 mV, each lasting 5 ms, are given a variable interval between the two pulses. The current during the second pulse [$I(t)$] is divided by the current during the first pulse [$I(\infty)$] is plotted against the recovery interval. Solutions: Na ASW (○) or Tris ASW (●)//200 Na.

QX314, 9AA, and *N*-methylstrychnine, but not for etidocaine and tetracaine. The traces on the left of Fig. 10 A illustrate 50% use-dependent block by QX222 of inward sodium currents brought about by 10 conditioning pulses to +110 mV with normal inactivation. This axon was then treated with pronase to eliminate sodium inactivation almost completely. The same pulse protocol resulted in no inhibition after pronase treatment; the control and test currents superimpose. This effect of pronase treatment on use-dependent inhibition is illustrated graphically for QX314 in Fig. 10 B. In the axon with unmodified inactivation, there is 85% modulation of the sodium current by conditioning pulses to variable potentials. In the pronase-treated axon, the same conditioning pulses produce relatively little modulation of the sodium current; there is little accumulation of block with repetitive pulsing. Pronase treatment also abolishes use-dependent block by strychnine (Cahalan and Shapiro, 1976) and by 9AA (Yeh and Narahashi, 1976).

Accumulating local anesthetic block with repetitive pulsing depends upon the slow time-course for recovery from block (see Fig. 2 B). After pronase treatment, the time-course for recovery from block can be measured in a two-pulse experiment for those compounds that induce time-dependent block, as shown for 9AA and etidocaine in Fig. 11. At the top left, an outward sodium current activates, reaches a peak, and then

is blocked by 9AA. After an interval of 0.5 ms, the same amplitude pulse is applied to reactivate the sodium current. The second peak of current sodium is nearly 80% of the first. This means that during the brief 0.5-ms repolarization, nearly 80% of the channels became unblocked. For comparison, in a normal axon with unmodified sodium inactivation gating, recovery from block by 9AA proceeds with a half time of about 6 s, more than four orders of magnitude slower. The bottom part of Fig. 10 A illustrates the time-course for recovery from 9AA block in a pronase-treated axon in the presence and absence of external sodium ions. External sodium ions slightly accelerate the recovery from block, which in either case is nearly complete within 1 ms. Pronase treatment abolishes use-dependent block by 9AA or by strychnine (Cahalan and Shapiro, 1976), because recovery from block is greatly accelerated.

After pronase treatment, recovery from block is still quite slow for etidocaine and tetracaine, in contrast to 9AA or strychnine. In the top trace of Fig. 10 B, the first voltage-clamp pulse activates a sodium current that then declines as etidocaine blocks the channels. After a 5-ms repolarization, a second voltage-clamp pulse results in much less sodium current than the first, indicating little recovery from block during the interval between the two pulses. The graph below indicates the time-course of recovery. The half-time for recovery from etidocaine block is on the order of 1 s. In similar two-pulse experiments with tetracaine, the half time for recovery in a pronase-treated axon was on the order of 400 ms. As a result some degree of use-dependent block by etidocaine and tetracaine persists at 1 Hz after pronase treatment.

*Local Anesthetic Effects on Sodium Tail Currents*

The two-pulse experiments on recovery from block in pronase-treated axons reveal a basic difference between compounds such as 9AA or *N*-methylstrychnine, which lose their use-dependent blocking after pronase treatment, and compounds such as etidocaine and tetracaine, which retain some degree of use dependence despite pronase treatment. Molecules such as QX222, QX314, 9AA, and strychnine evidently owe their use-dependent blocking in axons with normal inactivation to some channel component destroyed by pronase. Etidocaine and tetracaine, on the other hand, evidently remain in the blocked position after repolarization despite pronase treatment. In this section, the time-course of sodium tail currents is shown to be consistent with the idea that etidocaine and tetracaine remain in the channel upon repolarization, whereas blocking compounds such as 9AA or *N*-methylstrychnine rapidly leave the channels upon repolarization.

An experiment demonstrating the effects of 9AA on sodium tail currents is shown in Fig. 12. The axon was treated with pronase to remove sodium inactivation completely, and then perfused internally with a solution containing 170 $\mu$M 9AA. The outward sodium current in Fig. 12 A demonstrates the time-dependent block induced by 9AA for a depolarization to +70 mV. The inward currents represent tail currents at various times of repolarization. Tail currents for very brief depolarizations, before the decline of the outward sodium current, have the normal rapid exponential decline character-

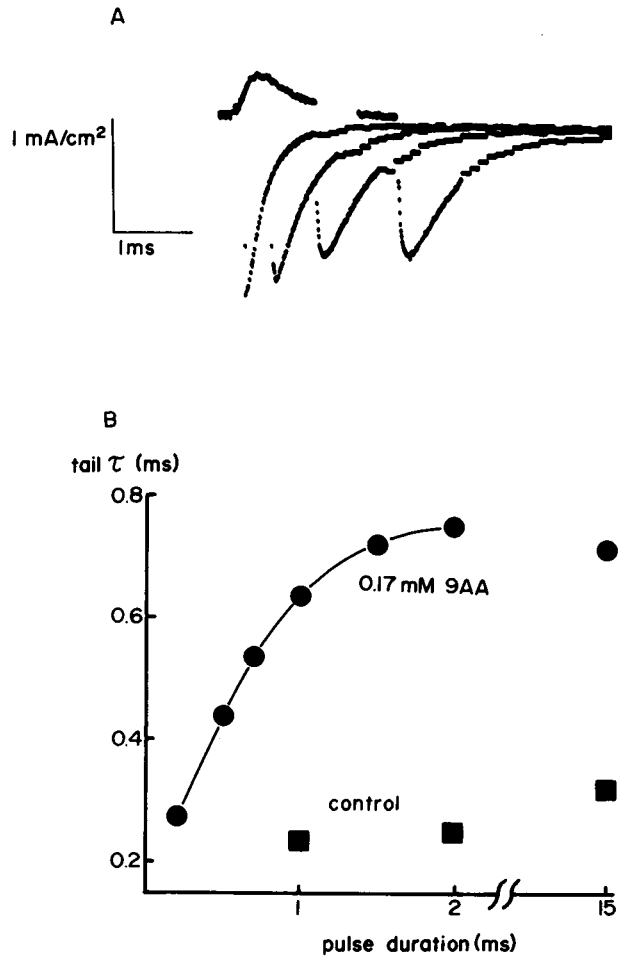



FIGURE 12 (A) Sodium currents with 9AA in pronase-treated axon, for variable pulse duration to +70 mV. (B) The time constants for sodium tail currents are plotted against the pulse duration before and after adding 9AA.

istic of activation gates rapidly closing upon repolarization. For longer pulses, the tail currents are progressively slower and become characterized by an initial increase in the inward sodium current upon repolarization. This type of effect, a "hook" in the tail current, is also observed with internal perfusion of pancuronium (Yeh and Narahashi, 1977) or strychnine (Cahalan and Shapiro, 1976). The tail currents become slower and develop hooks in parallel with the time-dependent block induced by 9AA. Tail currents after adding 9AA were fitted with exponentials during the later part of the current well after the hook. Fig. 12 B illustrates that the time constant ($\tau$) for sodium tail currents becomes slower for longer duration voltage-clamp pulses. For comparison, the time-constants for tail currents in a pronase-treated control axon

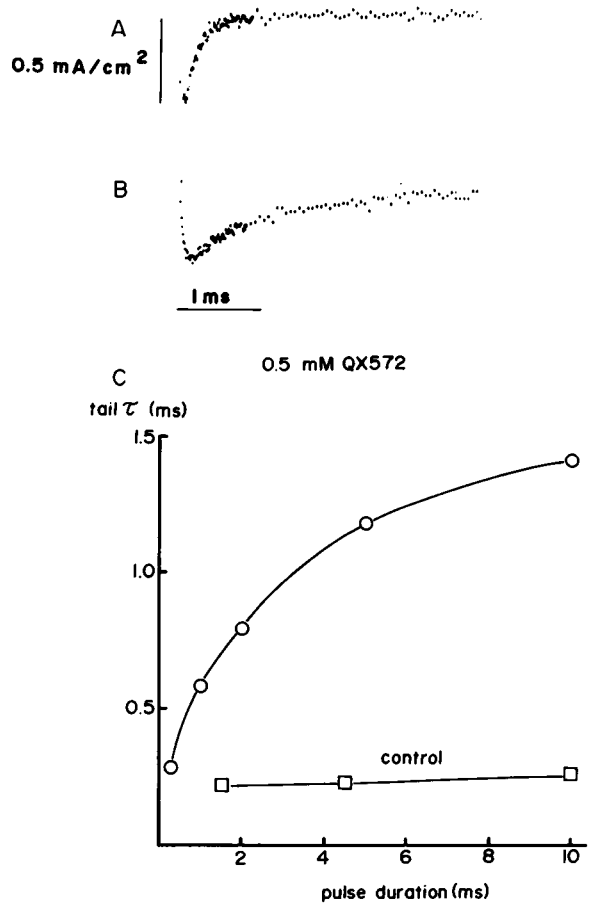



FIGURE 13 Sodium tail currents with QX572 in pronase-treated axon. (A) Tail current after a 0.4-ms depolarizing pulse to +90 mV. (B) Tail current after a 5-ms pulse to +90 mV. (C) Time constants for tail currents at variable pulse duration before (□) and after (○) adding QX572.

before adding 9AA are plotted showing a slight increase in $\tau$ with longer pulses. The time-constant upon repolarization increases by a factor of between three and four as block by 9AA progresses during the depolarization.

Fig. 13 illustrates the prolongation of tail currents by QX572. In A, a pulse of 0.2-ms duration is followed by a rapid exponential tail current. Fig. 13 B shows the tail current after a 5-ms depolarization; there is an initial hook and a slow decline to the base line. Fig. 13 C illustrates that for QX572, the tail currents decline slowly for longer duration pulses. For short duration pulses, the time-course of tail currents is nearly normal and becomes progressively longer as QX572 block develops with longer depolarizing pulses.

Fig. 14 illustrates tail currents in a pronase-treated axon for no drug added (control), for 2 mM procaine, and for 0.75 mM etidocaine. The left-hand traces illustrate tail

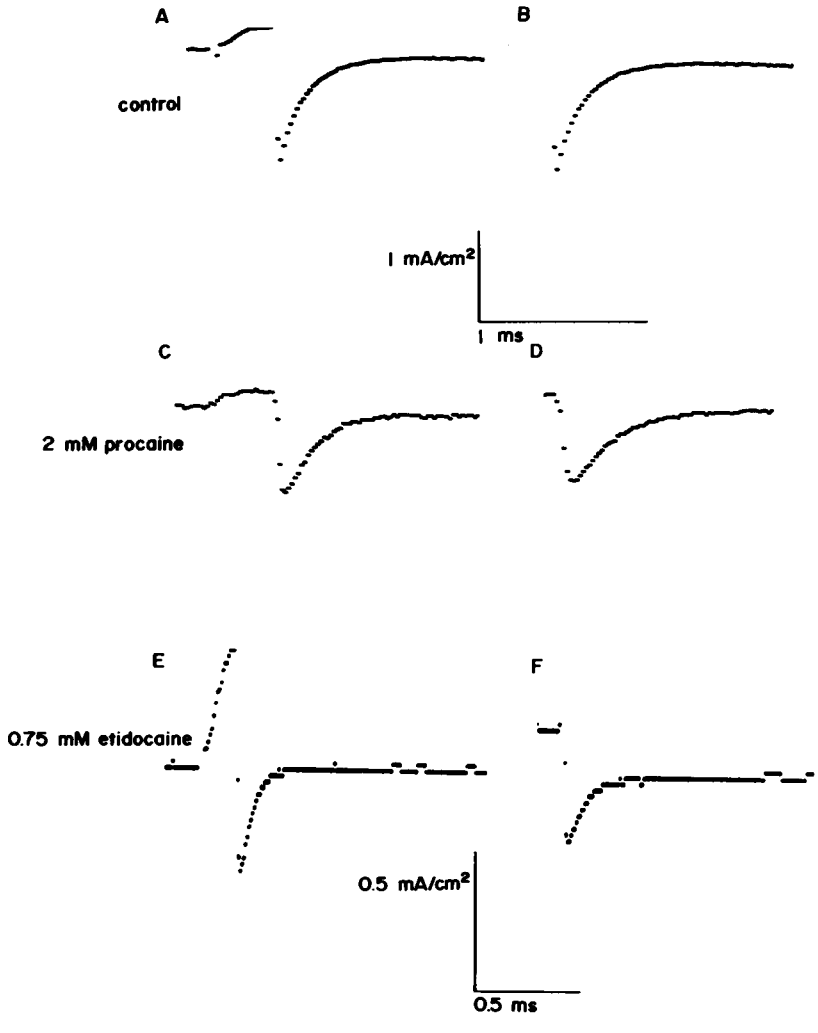



FIGURE 14 Sodium tail currents after pulse to 70 mV for 0.4 (left) or 5 ms (right). (A, B) Control tail currents in pronase-treated axon with no drug. (C, D) Same axon as A, B after adding 2 mM procaine inside. (E, F) Tail currents with 0.75 mM etidocaine inside. Solution: (A, B, C, D) Na ASW//50 Na SIS TEA; (E, F) Na ASW//200 Na.

currents for a brief depolarization, whereas the right-hand traces show tail currents for a 5-ms depolarization. The control traces show rapid tail currents with a rising phase of only one 10-$\mu$s sampling interval. With 2 mM procaine, the tail currents are altered even for a 0.4-ms pulse, reflecting the rapid blocking kinetics of procaine (see Fig. 7). The tail current has a hook lasting 50 $\mu$s on the left and 60 $\mu$s on the right. In the bottom traces with etidocaine inside the axon, the tail currents have a nearly exponential decline, though the time-course is slowed after the 5-ms pulse on the right. Even though there was >50% block by etidocaine during the depolarizing pulse, there is no hook in the tail current.

TABLE IV

CHARACTERISTICS OF BLOCK IN PRONASE-TREATED AXONS

| | Time-dependent block | Loss of use dependence | "Hook" in $I_{Na}$ tail |
|---|---|---|---|
| Procaine | Yes | — | Yes |
| Etidocaine | Yes | Slight | No |
| Tetracaine | Yes | Partial | No |
| NMS | Yes | Total | Yes |
| 9AA | Yes | Total | Yes |
| QX222 | No | Total | No |
| QX314 | No | Total | No |
| QX572 | Yes | — | Yes |

The hook in the tail current most likely represents drug molecules unblocking channels, resulting in a transiently increased sodium conductance until the activation gates close. This type of altered tail current is consistently observed with procaine, 9AA, QX572, strychnine, and *N*-methylstrychnine, but not with QX222, QX314, tetracaine, or etidocaine. Pronase treatment fails to abolish use-dependent block by tetracaine or etidocaine, because recovery from block is still slow. The hook in the tail current is probably absent because the time-course for unblocking with tetracaine and etidocaine is so slow. Pronase treatment abolishes both use-dependent and voltage-dependent block by QX222 and QX314. In this case, the lack of a hook in the sodium tail currents is probably a reflection of the fact that these two compounds do not participate in the voltage-dependent closed⟷open⟷blocked reaction after pronase

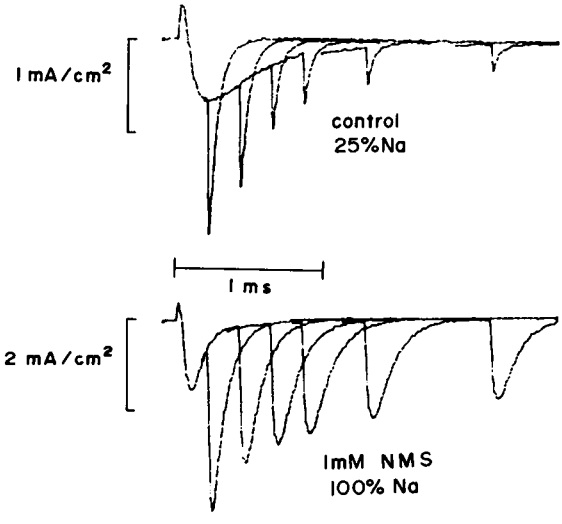



FIGURE 15 Sodium currents for variable pulse durations before (top) and after (bottom) adding 1 mM NMS in axon with unmodified sodium inactivation. Solution: (top) 25% Na ASW, 75% Tris ASW//SIS TEA, (bottom) Na ASW//SIS TEA.

treatment. Table IV summarizes some of the blocking characteristics induced by the eight compounds studied in pronase-treated axons.

One additional type of drug interaction with sodium channel gating is the prevention of the development of inactivation that occurs with pancuronium (see Fig. 9 of Yeh and Narahashi, 1977) and with *N*-methylstrychnine (NMS), as illustrated in Fig. 15. In the top trace, the time-course of normal sodium inactivation, $h$, can be seen both in the decline of the inward sodium current during a maintained depolarization and in the decreasing amplitude of sodium tail currents upon repolarizing after pulses of increasing duration. The peak amplitude of tail currents parallels the time-course of inactivation. These records were obtained with 25% normal sodium in the external solution; the ionic currents are therefore reduced, and sodium gating currents can be seen in the early outward current and in the late inward tail of current after inactivation is complete. The bottom trace illustrates currents for the same pulse protocol, interrupting a depolarizing pulse at variable times, with 1 mM NMS inside the axon. The external sodium was raised to 100% (440 mM) of normal artificial sea water to increase the inward currents, partially offsetting the decline of sodium current amplitude produced by NMS. During the depolarization, the sodium current reaches a peak of inward current and then declines more rapidly than in the control traces. This faster "inactivation" of $I_{Na}$ probably represents NMS molecules entering and blocking open sodium channels more rapidly than the inactivation process. If inactivation proceeds with a normal time-course, the tail currents for long depolarizing pulses should be small. However, there is a large tail of inward current even for the longer depolarizing pulses, indicating that NMS interferes with the normal development of sodium inactivation. The amplitude of tail currents no longer parallels the time-course of the sodium current during a maintained depolarization. Evidently, NMS interferes or competes with the inactivation mechanism. These tail currents also exhibit hooks reflecting the unblocking by NMS that must occur before activation gates close. Thus, when NMS has blocked a channel, both activation and inactivation gating are impeded.

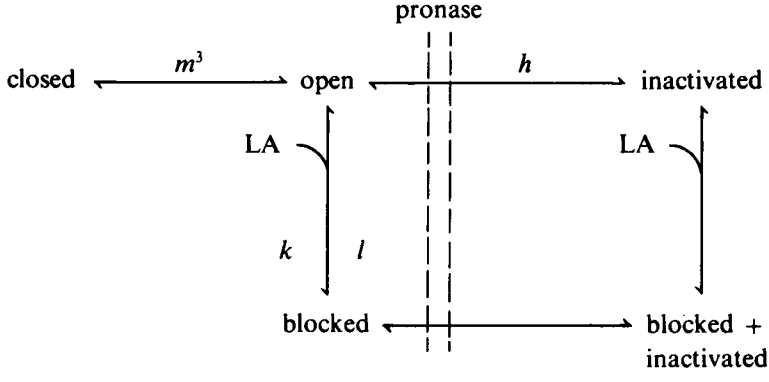
## DISCUSSION

### *Use-Dependent Block Requires Sodium Inactivation*

Except for abstracts (Cahalan and Shapiro, 1976; Yeh and Narahashi, 1976; Lipicky et al., 1977; Almers and Cahalan, 1977), this is the first description of use-dependent local anesthetic block of sodium currents in squid giant axon. Use-dependent block has been carefully studied previously both in frog myelinated nerve and skeletal muscle fibers (Strichartz, 1973; Courtney, 1975; Khodorov et al., 1976; Hille, 1977*a*, *b*; Schwarz et al., 1977). The features of local anesthetic block in squid axon are quite similar: block accumulates with repetitive pulsing at low frequencies; the time-course of sodium current during each pulse is not greatly altered with respect to control, except for strychnine and NMS (see Fig. 15); recovery from block at the resting potential is slow; the degree of inhibition depends upon pulse frequency and membrane potential; and inactivated channels are less susceptible to block. In the squid axon, use-depen-

dent block produced by any of the seven compounds studied (procaine was not included) has very similar characteristics with differences between compounds appearing mainly in the time-course for recovery from block (see Table I). The voltage dependence for block established with repetitive pulsing is slightly less than an *e*-fold change in block per 24.6 mV, the expected value for a single charge moving through the membrane field.

The experiments presented here provide support for a direct role of the sodium inactivation gating mechanism, *h*, in producing the long lasting, use-dependent inhibition. The role of sodium inactivation in local anesthetic block was initially suggested by Weidmann (1955), and recently supported in experiments on myelinated nerve fibers (Courtney, 1975; Khodorov et al., 1976; Hille, 1977*b*). Removing the inactivation process with pronase inside a squid axon abolishes use-dependent block by QX222, QX314, strychnine, and 9AA. Thus, use-dependent block by these compounds appears to depend on a slow, possibly voltage-dependent binding and unbinding to a channel component attacked by pronase. It seems likely that this component is the inactivation gating mechanism, though multiple effects of pronase upon the channel cannot be definitely excluded. In terms of a kinetic diagram, pronase treatment would prevent the channel from reaching a new state, called blocked and inactivated:




This diagram is closely related to the kinetic models described by Courtney (1975) and by Hille (1977*b*) in ascribing a central role to normal sodium inactivation.

In the diagram above, an open channel can be either blocked by local anesthetic (LA) or inactivated by the *h* process. A blocked channel in turn can interact with the inactivation mechanism to reach the blocked and inactivated state. Access to this state from inactivated channels is probably slower than from blocked channels, because inactivated channels are relatively resistant to use-dependent block in conditioning pulse experiments (see Strichartz, 1973; and Fig. 3). Recovery from the blocked and inactivated state is very slow, having the time-course measured in Fig. 2 B. After pronase treatment, the blocked and inactivated state cannot be reached, and recovery from block by most compounds is a relatively rapid process. Two of the compounds studied, tetracaine and etidocaine, still possess some degree of use dependence after pronase treatment, reflecting an intrinsically slow transition from the blocked state to

the closed state. There may, in addition to the states diagramed above, be a closed and blocked state for etidocaine and tetracaine. Compounds such as *N*-methylstrychnine, procaine, QX572, and 9AA may lack the closed and blocked state, for the altered time-course of sodium tail currents suggests that activation gates cannot close on blocked channels.

*Comparison Between Blocking Compounds*

In pronase-treated axons, the general family of compounds that induce use-dependent block in axons with normal inactivation gating may be subdivided into a variety of effects. All, except for QX222 and QX314, result in time-dependent block of sodium currents during a maintained depolarization. Compounds such as 9AA and etidocaine exhibit significant voltage dependence of block during a single pulse. In contrast, for QX222 and QX314 inside a pronase-treated axon, the voltage dependence is not apparent in individual pulses. It seems likely that the voltage dependence of block is not simply a result of the movement of the blocking ion to and from the blocking site within the membrane, as is imagined for the block of sodium channels by hydrogen ions (Woodhull), 1973) or calcium ions (Taylor et al., 1976). After pronase treatment, compounds such as 9AA and etidocaine do retain an intrinsic voltage dependence for the block established with a single pulse. However, QX314 and QX222 lose this voltage dependence, and the voltage dependence observed with strychnine (Cahalan and Shapiro, 1976) and to some extent 9AA can be modified by altering the sodium concentration gradients. Furthermore, the block of the $I_{Na}$ by calcium ions has a voltage dependence placing the Ca site about half-way across the membrane electric field (Taylor et al., 1976), and it is difficult to imagine local anesthetic molecules on the inside moving 90% of the way across the field beyond a Ca binding site. It is perhaps more reasonable to assign the voltage dependence observed with conditioning pulse experiments in axons with normal gating to the interaction with the inactivation mechanism forming the inactivated and blocked state. This would help to reconcile the relatively uniform voltage dependence of all of the compounds studied in normal axons (see Table II) with the variety of effects by different compounds observed in pronase-treated axons.

Etidocaine and tetracaine retain some degree of use dependence after pronase treatment, because recovery from block is still slow. Strychnine, 9AA, QX222, and QX314 lack use dependence in pronase-treated axons, and for 9AA and strychnine, the time-course for recovery from block is accelerated by about four orders of magnitude relative to recovery in axons with normal inactivation.

In a pronase-treated axon, the block produced by local anesthetic compounds greatly resembles the block of potassium channels by TEA derivatives studied by Armstrong (1969). A time-dependent "gating" process is induced by the blocking compound from the axoplasmic side of the membrane. The channel must be open for block to occur. In terms of channel properties, these results suggest that both sodium and potassium channels have a relatively wider inner region accessible to blocking molecules in axoplasm after the activation process opens the channel. Recovery from

block can be rapid and altered by external permeant ions, though the interaction with ionic current seems to be more pronounced for potassium channel block. In pronase-treated axons, pancuronium ions (Yeh and Narahashi, 1977) and TEA derivatives (Rojas and Rudy, 1976) also induce time-dependent blockage of the sodium channels. However, these compounds apparently do not produce use-dependent block in axons with normal sodium inactivation gating.

These and other blocking compounds should prove to be useful probes of the sodium channel. A variety of interactions with sodium channel gating have been demonstrated. Perhaps the anesthetic receptor site inside the channel that is made available when the channel activates is also the receptor for an inactivation gating particle attacked by pronase. Both local anesthetics and the inactivation mechanism block current through the activated channel. At least QX314 (Almers and Cahalan, 1977) and NMS[1] immobilize gating charge movement associated with channel activation, as does the normal inactivation process (Armstrong and Bezanilla, 1977). In addition, NMS (see Fig. 15) and pancuronium ions (see Yeh and Narahashi, 1977) evidently compete with the normal inactivation mechanism, preventing inactivation while blocking the channel. As more is learned about which compounds exhibit different types of gating interaction, structure-activity relations may provide clues to the molecular organization of sodium channels. By studying the properties of artificial channel gates, we hope our picture of the natural gating mechanism will become clearer.

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