

Inhibition of Sodium Currents by Local Anesthetics in Chloramine-T-treated Squid Axons

The Role of Channel Activation

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ABSTRACT In order to test the requirement of Na channel inactivation for the action of local anesthetics, we investigated the inhibitory effects of quaternary and tertiary amine anesthetics on normally inactivating and noninactivating Na currents in squid axons under voltage clamp. Either the enzymatic mixture pronase, or chloramine-T (CT), a noncleaving, oxidizing reagent, was used to abolish Na channel inactivation. We found that both the local anesthetics QX-314 and etidocaine, when perfused internally at 1 mM, elicited a "tonic" (resting) block of Na currents, a "time-dependent" block that increased during single depolarizations, and a "use-dependent" (phasic) block that accumulated as a result of repetitive depolarizations. All three effects occurred in both control and CT-treated axons. As in previous reports, little time-dependent or phasic block by QX-314 appeared in pronase-treated axons, although tonic block remained. Time-dependent block was greatest and fastest at large depolarizations (E_m greater than +60 mV) for both the control and CT-treated axons. The recovery kinetics from phasic block were the same in control and CT-modified axons. The voltage dependence of the steady state phasic block in CT-treated axons differed from that in the controls; an 8–10% reduction of the maximum phasic block and a steepening and shift of the voltage dependence in the hyperpolarizing direction resulted from CT treatment. The results show that these anesthetics can bind rapidly to open Na channels in a voltage-dependent manner, with no requirement for fast inactivation. We propose that the rapid phasic blocking reactions in nerve are consequences primarily of channel activation, mediated by binding of anesthetics to open channels, and that the voltage dependence of phasic block arises directly from that of channel activation.

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INTRODUCTION

Local anesthetics are a class of drugs that reversibly block nerve conduction. Despite the long history of clinical use of these chemicals, the precise mechanism(s) of local anesthetic action on Na channels is still unsettled. During the last decade, a modulated receptor hypothesis for the interactions of local anesthetics and Na channels (Khodorov et al., 1976; Hille, 1977*b*; Hondeghem and Katzung, 1977) has gained strong experimental support and provides a clear framework for further experiments.

According to Hille (1977*a, b*), all local anesthetic molecules bind to a common receptor in the channel that can be reached by a hydrophilic or hydrophobic pathway. In addition, this common receptor is proposed to have different configurations because of structural rearrangements of the Na channel during depolarization. Specifically, the local anesthetic molecule binds most strongly to the inactivated state and thereby stabilizes the inactivated form of the channel. The "resting" form of the channel can equilibrate with the local anesthetic molecules, but with lower affinity than the inactivated form. During repeated depolarizations, Na channels accumulate in an inactivated form, being maintained in that conformation by the local anesthetic, thus accounting for the widely observed "use-dependent" behavior (Strichartz, 1973; Courtney, 1975; Khodorov et al., 1976; Schwarz et al., 1977). This hypothesis explains both the tonic blocking effect of local anesthetics, which occurs at rest, and the use-dependent inhibition. The core of Hille's hypothesis states that "the reaction of drug with one form may also have different rates and equilibria from the reaction with another form" (Hille, 1977*b*). Unfortunately, the kinetic descriptions of these rates and equilibria are not unique and can be interpreted in several ways (e.g., Khodorov et al., 1976; Shepley et al., 1983; Starmer et al., 1984; Yeh and Tanguy, 1985; Strichartz and Wang, 1986). For example, Khodorov et al. (1976) suggested that the slow inactivation process is directly involved in the local anesthetic binding interaction, whereas Starmer et al. (1984) theorized that a transiently accessible binding site with a constant binding affinity accounts for use-dependent block.

Because of this ambiguity, pharmacological modifications of specific gating processes are desirable for distinguishing between the alternatives. In fact, the strongest evidence for Hille's hypothesis is from the modification experiments of Cahalan (1978) and Yeh (1978). Independently they found that after removal of Na channel inactivation by pronase, the drug QX-314, a quaternary lidocaine derivative, was no longer capable of producing significant use-dependent block. They concluded that the inactivation mechanism plays a direct role in the actions of local anesthetics. Additional evidence was later provided by Bean et al. (1983), who showed that lidocaine bound much more strongly to cardiac Purkinje fiber Na channels after prolonged depolarization. The increase in lidocaine's binding affinity was estimated to be ~45-fold, being 440 μ M for the resting channels and 10 μ M for the inactivated channels.

In contrast, the role of the activation process in the binding of local anesthetics is not specifically defined in Hille's hypothesis, although Strichartz (1973) and

Hille (1977*b*) have demonstrated that the charged local anesthetics can only interact with the Na channel in its open conformation. Therefore, the activation "gate" seems to limit the access and the escape of the charged local anesthetic from its site on the channel. Recently, a direct involvement of the channel activation mechanism in local anesthetic action was proposed (G. K. Wang and Strichartz, 1984; Yeh and Tanguy, 1985; Strichartz and Wang, 1986). The purposes of this report are to define the role of the inactivation and activation processes in the inhibitory action of local anesthetics on Na currents in squid giant axons and to re-examine the kinetic details of the modulated receptor hypothesis.

The word "inhibition" is used interchangeably with "block" in the text and, unless otherwise noted, implies no physical mechanism. We specifically chose a mild oxidant, chloramine-T, to modify the inactivation process (G. K. Wang et al., 1985). The advantages of using chloramine-T are twofold: (a) unlike pronase, it does not cleave the primary peptide bonds of proteins, and (b) like pronase, it appears to have no significant effects on the Na channel activation process.

MATERIALS AND METHODS

Voltage Clamp of Squid Giant Axons

Squid (*Loligo pealei*) were supplied by the Marine Biological Laboratory, Woods Hole, MA. Squid giant axons (~400–650 μm diam) were dissected, cannulated, and briefly exposed to 60 μg pronase (Calbiochem-Behring Corp., La Jolla, CA) per milliliter of internal perfusate for <5 min. The brief exposure to a low concentration of pronase allowed the axons to be perfused more easily, without significantly affecting the Na current kinetics. The axons were then perfused with standard internal perfusate (SIP) alone and voltage-clamped as described previously (Wu and Narahashi, 1973). The reference electrode was made of an agar bridge containing 0.5 M KCl. In some axons, pronase at 1 mg/ml was applied to remove the Na channel inactivation (Armstrong et al., 1973). The reaction was stopped after ~10 min by perfusing the axons with SIP alone. All experiments were performed at $9.5 \pm 1.0^\circ\text{C}$.

Leakage currents and capacitive transients were subtracted by an analog circuit. The current records were digitized at various rates (usually 10 μs /point) using a digital oscilloscope (model 206, Nicolet Instrument Corp., Madison, WI) and stored on magnetic disks. Current traces were later displayed on the oscilloscope and photographed. Many of the figures shown in this report contain multiple current traces. Only two current traces in each figure are from original records; additional current traces (except tail current traces, unless otherwise indicated) were drawn according to the original records. For kinetic analysis, the amplitudes of the photographed current traces were digitized using a Digiplot (Houston Instrument Co., Austin, TX) in conjunction with an eight-bit micro-computer (Horizon 2, North Star Computers, San Leandro, CA).

Solutions

The external artificial seawater (ASW) contained 440 mM NaCl, 10 mM KCl, 50 mM CaCl_2 , and 10 mM HEPES, pH 7.2. The SIP contained 325 mM K glutamate, 50 mM NaF, 333 mM sucrose, 25 mM tetraethylammonium bromide, and 20 mM HEPES, pH 7.3.

Chemicals

Chloramine-T was purchased from Fisher Scientific Co., Pittsburgh, PA, and dissolved in solution just before use. The internal exposure with chloramine-T at 7.1 mM was limited to periods of up to 15 min, since longer exposures might have nonspecific effects on the activation process. QX-314 and etidocaine were gifts from Dr. Bertil Takman of Astra Pharmaceutical Products, Inc., Worcester, MA. Tetrodotoxin was obtained from Calbiochem-Behring Corp. All other chemicals were reagent grade from commercial sources.

RESULTS

We have studied the interaction of two local anesthetics, QX-314 and etidocaine, with Na channels in squid axons. QX-314 and etidocaine contain a quaternary and a tertiary amine, respectively (Fig. 1). Since the kinetics and voltage dependence of Na channel block by these local anesthetics in squid axons have been described previously, we will first briefly report our similar results. Then we will

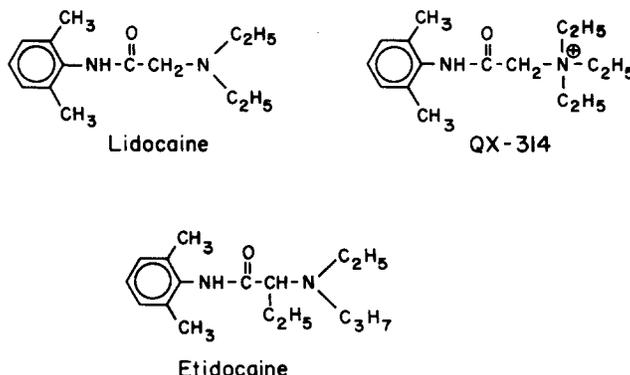


FIGURE 1. Chemical structures of QX-314, etidocaine, and lidocaine. QX-314, a quaternary lidocaine derivative, passes through the membrane very slowly and is active only when applied internally.

compare these results with new findings in axons pretreated with chloramine-T (CT). The general effects of QX-314 on Na currents in control axons and in CT-treated axons stimulated at low frequency (≤ 1 stimulus/30 s) are described in detail, interspersed with a shorter description of the very similar effects of etidocaine. In the remainder of this section, we present the use-dependent actions of local anesthetics measured at a higher stimulus frequency (1 Hz).

QX-314 and Etidocaine Modulate the Steady State Na Currents during a Single Strong Depolarization in Control Squid Axons

Local anesthetics, such as QX-314 at 1 mM in the axoplasmic compartment, reduced the Na current amplitude by up to 30%, but did not alter the kinetics of inward Na currents in axons with intact inactivation (Fig. 2, inward currents; also see Cahalan, 1978, and Yeh, 1978). The time course of the inward Na current decline was accelerated by QX-314, but only slightly (Fig. 3A). However, very different results were obtained at large depolarizations. Na channel inacti-

vation has been reported to be incomplete in squid axons at large depolarizations (Chandler and Meves, 1970). Under our experimental conditions, a maintained current was evident in the control perfused axons at the end of a 16-ms pulse to potentials greater than +60 mV. The kinetics of outward Na currents during such large depolarizations were modified by QX-314 in two respects (Fig. 2). First, the relative reductions of peak and steady state currents differed; in general, the steady state currents were inhibited more than the peak currents by QX-314. This phenomenon has been noted previously (Yeh, 1978). Second, the Na current declined with a multiexponential time course (Fig. 3B). The fast declining phase was like that of the control, but a second, slowly declining phase

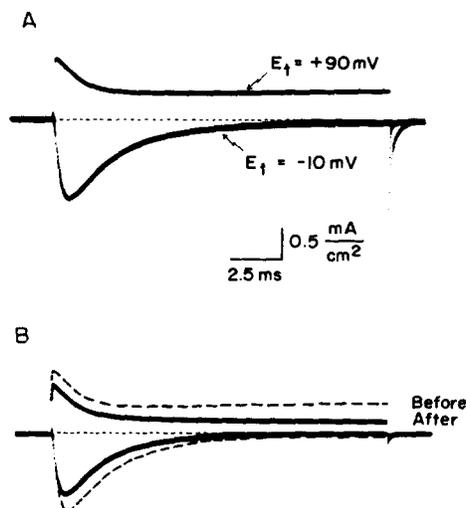


FIGURE 2. Na currents before (A) and after (B) the internal application of 1 mM QX-314. Both inward and outward Na currents are shown, corresponding to membrane test potentials (E_t) of -10 and $+90$ mV, respectively. The holding potential (E_H) was -70 mV. The peak current amplitudes in A at $E_t = -10$ and $+90$ mV are reduced in B by 20 and 17%, respectively. For comparison, the current traces before the local anesthetic treatment are included in B as dashed lines, except for the tail currents.

became evident, and appeared as a time-dependent reduction in the steady state currents. This phenomenon implies that QX-314 interacts slowly with the open Na channel during a single strong depolarization. However, a modicum of maintained current was always present with 1 mM QX-314 after a single, prolonged pulse or several repetitive depolarizations. The nature of this current component will be described later.

The tertiary amine local anesthetic etidocaine produced essentially identical results as those of the quaternary amine QX-314. For example, like QX-314, etidocaine produced both a tonic block of the peak Na current and a time-dependent block of the maintained current. Again, the fast inactivation time course was not much changed or was only slightly accelerated, but a slower

declining phase was evident at large depolarizations. The kinetics of this current inhibition were similar to those produced by QX-314 (Fig. 3B), but were slow compared with the normal fast inactivation process.

QX-314 and Etidocaine Modulate the Na Currents in Axons Pretreated with CT

Na channel inactivation is inhibited by CT as effectively as it is by pronase. More than 75% of the peak Na current remained at the end of a 20-ms pulse to -10 mV after 10–15 min of exposure to CT. These effects were irreversible and the

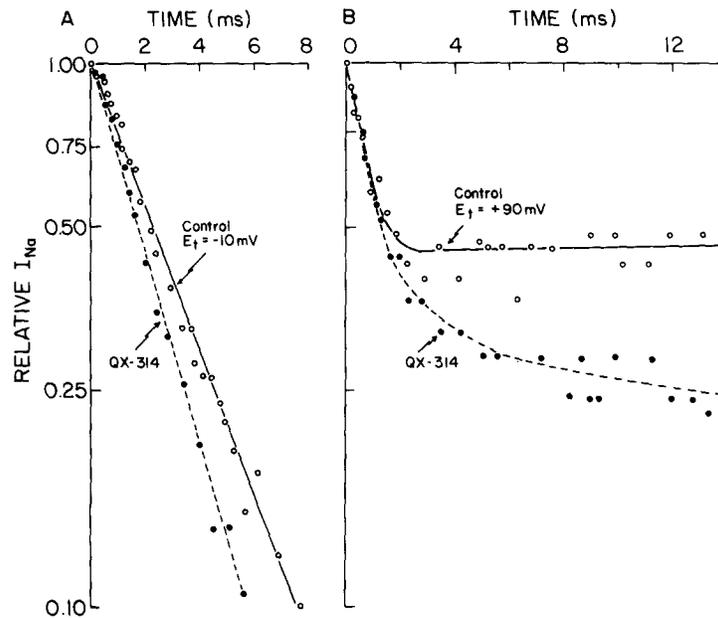


FIGURE 3. The time course of Na channel inactivation before (open circles) and after (filled circles) the internal application of 1 mM QX-314. In A, the time course was measured at $E_m = -10$ mV. The current that remained at various times during the declining phase was normalized to the peak current amplitude and plotted on a semilog scale against time, with time zero set at the time of peak current. In B, the time course was measured at $E_t = +90$ mV. At steady state, there is a residual current, equal to 45% of the peak inward current before the internal application of QX-314, which is reduced by QX-314 to <25% of peak current after a 14-ms depolarization. The slowly decaying component has a half-time of ~ 3 ms (data from Fig. 2).

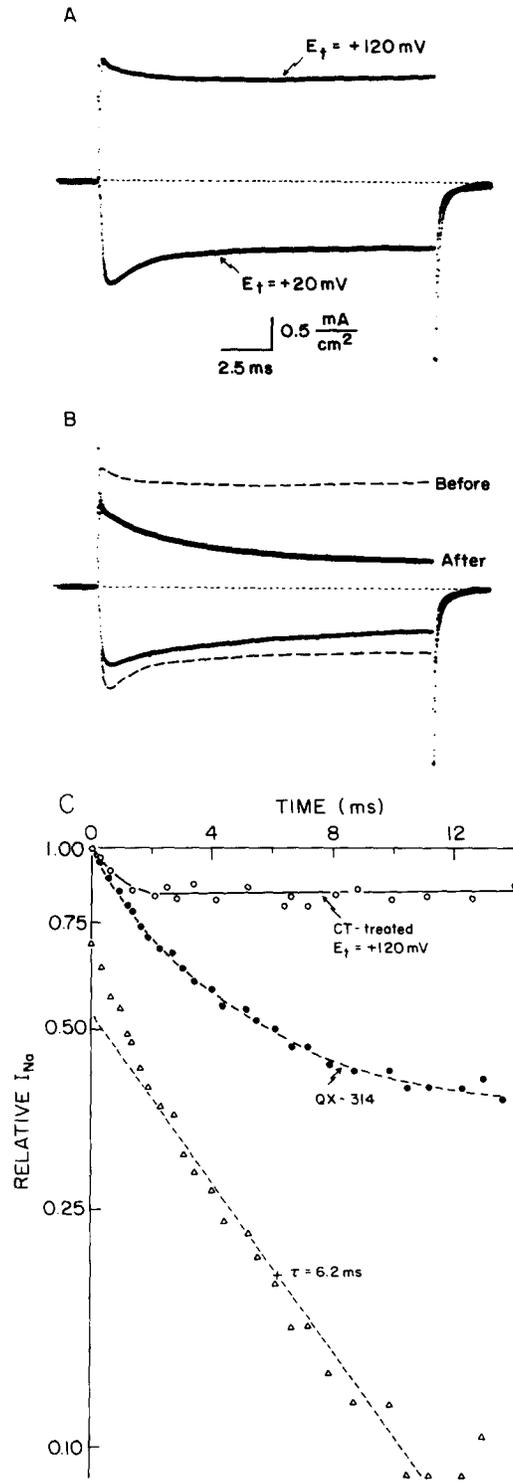
reaction could be stopped by washing. In some axons, we noticed that residual inactivation required prolonged treatment with CT. To limit the deterioration of axons and any effect of CT on channel activation, we generally treated the axons internally for <15 min at a concentration of 7.1 mM (2 mg/ml). As a result, a small degree of inactivation ($\leq 25\%$ at $E_m = -10$ mV) was present in some of the axons we studied, although the effects of the anesthetics on current in these preparations were identical to those in axons with no detectable inactivation.

When QX-314 at 1 mM was applied internally to the CT-treated axons, both the tonic block of the peak Na currents and the time-dependent block of maintained currents could be observed at large depolarizations (Fig. 4, *A* and *B*). In the CT-treated axon shown in Fig. 4, modest inactivation of Na currents was present before QX-314 treatment. After the addition of local anesthetics, a time-dependent inhibition of the maintained outward Na currents appeared (Fig. 4*C*), with kinetics comparable to those in the control axons exposed to anesthetic (e.g., Fig. 3). At small depolarizations, the time-dependent reduction by QX-314 of Na currents in CT-treated axons was smaller and much slower (Fig. 4, *B* and *C*) than at large depolarizations, despite the presence of some residual inactivation at small depolarizations before anesthetic addition. In the experiment illustrated in Fig. 4, the inhibition of peak and maintained currents by 1 mM QX-314 was 26 and 42% at $E_m = +20$ mV, and 32 and 75% at $E_m = +120$ mV, respectively. The time-dependent inhibition that developed at small depolarizations varied considerably from axon to axon for reasons unknown to us. However, the difference between the inhibitions of peak and maintained currents rarely exceeded >20% at E_m greater than +20 mV with a pulse duration of <20 ms (also see Fig. 10*A*), which shows that the time-dependent block is weak for such small potentials. Thus, local anesthetics appear to interact with the open channel in a voltage-dependent manner. For large depolarizations, the inhibition by QX-314 is evident from the slow decline of previously "noninactivating" currents (Fig. 5). When corrected for the pre-existing current droop, the subsequent addition of drug caused a time-dependent block that was both larger and faster at greater depolarizations.

Similar results were found for etidocaine in the CT-treated axons (Fig. 6). A strong time-dependent block, developing with a time constant of 8.0 ms, was detected at large depolarizations (+80 mV), but a weaker and slower inhibition occurred at smaller depolarizations. These results demonstrate that CT treatment does not significantly alter the action of the local anesthetics. In contrast, in axons treated with pronase (1 mg/ml for ~10 min), QX-314 produced only a minor time-dependent reduction of Na currents, even at large depolarizations (see Yeh, 1978). Interestingly, etidocaine (Cahalan, 1978) and the spin-labeled local anesthetic C6SLMEI (H. H. Wang et al., 1982) continue to produce a time-dependent block of Na currents at large depolarizations in pronase-treated axons. These different and sometimes contradictory results for the pronase-treated and CT-treated axons indicate that chemical modifications can cause a selective loss of the otherwise identical inhibitory effects caused by different local anesthetics.

Phasic Block of Na Currents by Local Anesthetics in Normal Axons

Repetitive depolarizations produced additional block of Na currents in the QX-314-perfused squid axons, as described previously (Cahalan, 1978; Yeh, 1978). This effect, here referred to as phasic (use-dependent) block, also depended on the magnitude of the applied depolarization. Fig. 7*B* shows that small repetitive depolarizations ($E_m = -10$ mV), applied at 1 Hz for a total of 10 pulses, reduced I_{Na} by ~25% but had little effect on the kinetics of I_{Na} . Such a phasic block reached steady state within the first 10 pulses; additional pulses produced no further inhibition. In contrast, strong repetitive depolarizations ($E_m = +90$ mV)



produced a larger block and affected the kinetics of Na currents during the pulse as well (Fig. 7A). The peak current was reduced dramatically at the 10th depolarization, whereas the maintained current was relatively unaffected. Consequently, most of the current remaining at the 10th pulse appears to be noninactivating. Similar changes in current kinetics by repeated strong depolarizations have also been observed in control axons perfused with 9-aminoacridine (Yeh and Oxford, 1985).

When tested by currents at smaller depolarizations, the change in I_{Na} kinetics induced by large conditioning pulses was still apparent. Fig. 7C shows currents

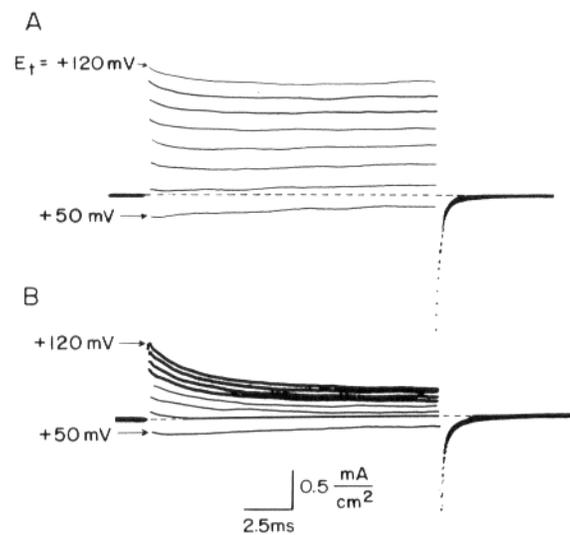


FIGURE 5. The time-dependent block by QX-314 (1 mM) in a CT-treated axon at various large depolarizations (B) and the control currents (A). The test membrane potential was increased by 10 mV for each current trace starting from +50 to +120 mV. The interval between each depolarization was 1 min. $E_H = -70$ mV.

FIGURE 4. (*opposite*) The effect of QX-314 on Na currents in a CT-treated axon. Inward and outward Na currents at +20 and +120 mV, respectively, are shown in A after CT treatment. In B, the peak inward and outward Na currents were reduced by 26 and 32%, respectively, by internal QX-314 (1 mM). Note the large time-dependent block of outward Na current. The declining phases of outward Na currents in A and B were analyzed, normalized to the peak current amplitude, and plotted against time in graph C. The open and filled circles represent the time course before and after internal QX-314 application. The triangles show the currents in QX-314 (filled circles) after subtraction of the maintained Na current. A slow component was fitted by eye to these points (dotted line with a time constant of 6.2 ms). The decline of inward Na currents is not shown, but the time-dependent block was significantly smaller than for the outward Na currents in this axon and in five other axons under similar conditions. Time zero indicates the time of peak Na current amplitude.

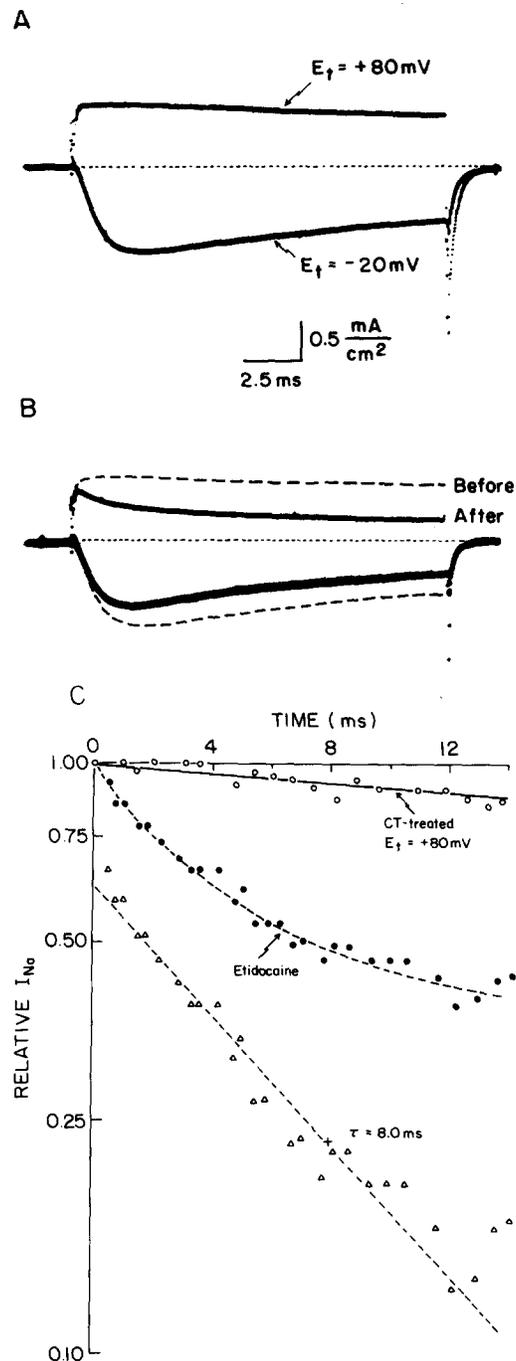


FIGURE 6. The effect of etidocaine on Na currents in a CT-treated axon. Inward and outward Na currents were measured at -20 and $+80$ mV, respectively, before (A) and after (B) the internal application of 1 mM etidocaine. (C) The time-dependent block by etidocaine of the outward Na current at $+80$ mV.

at -20 mV before and after conditioning by $+90$ mV pulses. The relative decline from the peak current value observed at the end of the test pulse was 85–90% before conditioning and 60–70% after conditioning. As in Fig. 10A (see below), conditioning appears to selectively depress the peak currents.

The complete reversal of phasic block by QX-314 usually took 1–2 min in resting axons, with a half-recovery time of ~ 10 –20 s, which is comparable to the

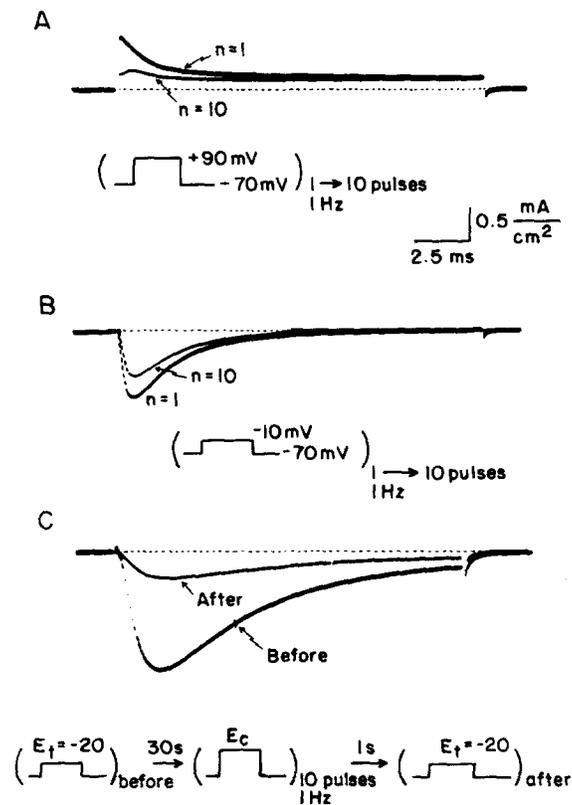


FIGURE 7. QX-314 modifies the current kinetics in a control axon during strong repetitive pulses. The outward currents at $E_t = +90$ mV are shown in *A* before and after nine pulses applied at 1 Hz. In *B*, the inward Na currents at $E_t = -10$ mV were measured before and after nine pulses at 1 Hz. In *C*, the Na current at $E_t = -20$ mV was before and 1 s after conditioning by large depolarizations (10 pulses to $E_c = +90$ mV for 16 ms at 1 Hz). The pulse protocol is shown below the current traces. The data in *A* and *B* were obtained from the same axon, whereas in *C*, a different axon was used.

14.5 s reported for squid by Cahalan (1978). This slow recovery permits the measurement of the voltage dependence of phasic block using a protocol like that in Fig. 7C. Steady state phasic block is strongly dependent on the magnitude of the conditioning pulses (Fig. 8A). More than 75% of Na currents could be blocked by conditioning potentials greater than $+50$ mV. In contrast, when the axons were pretreated with pronase, QX-314 no longer produced a time-

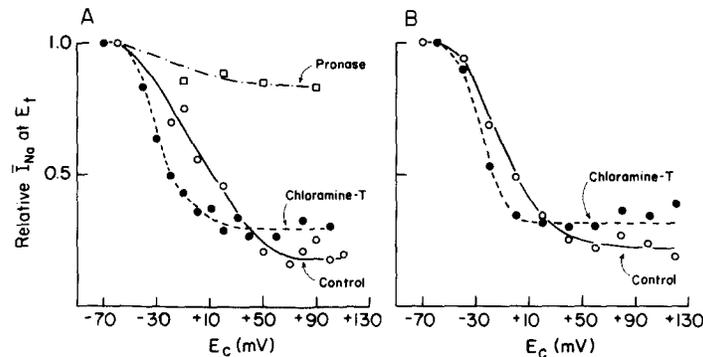


FIGURE 8. Voltage dependence of steady state use-dependent block of Na currents by local anesthetics in control, CT-treated, or pronase-treated squid axons. (A) A CT-treated axon (filled circles), a control axon (open circles), and a pronase-treated axon (open squares), each containing 1 mM QX-314, were depolarized by 10 repetitive conditioning pulses (E_c) at 1 Hz. The relative peak Na currents remaining at $E_t = 0$ mV were normalized by the value before conditioning and plotted against E_c . (B) The same analysis was performed on a CT-treated axon (filled circles) and a control axon (open circles), both perfused internally with 1 mM etidocaine. $E_H = -70$ mV.

dependent inhibition and phasic block was greatly diminished ($\leq 20\%$ block; see Table I and Fig. 8A, squares). This result confirms observations reported previously by Cahalan (1978) and Yeh (1978).

Like QX-314, etidocaine also produced a phasic block of Na currents in normal axons (data not shown). The voltage dependence of this block is shown in Fig. 8B. Thus, quaternary and tertiary amine local anesthetics behave almost identically in squid axons, although the half-time for recovery from phasic block is faster in etidocaine-treated axons than in QX-314-treated axons (3.0 vs. 14.5 s, respectively; Cahalan, 1978).

TABLE I
Summary of the Effects of QX-314 and Etidocaine on Na Currents in the Normal and the Chemically Modified Squid Axons

Phenomenon	Control*		CT-treated		Pronase-treated	
	QX-314	Etidocaine	QX-314	Etidocaine	QX-314*	Etidocaine [‡]
Tonic block	+	+	+	+	+	+
Time-dependent block						
At large depolarizations (E_t greater than +60 mV)	+	+	+	+	Little	+
At small depolarizations (E_t less than +40 mV)	-	-	(+)§	(+)	-	-
Use-dependent block	+	+	+	+	Little	ND

* From this study and from Cahalan (1978) and Yeh (1978).

[‡] From Cahalan (1978).

§ Positive but less significant than at large depolarization. ND, not determined.

Use-dependent Block in CT-treated Axons

As demonstrated in this report, QX-314 and etidocaine produced a tonic and a time-dependent block of Na currents at large depolarizations in CT-treated axons. Unexpectedly, they also produced a significant phasic inhibition in such axons, almost equal to that in unmodified nerves and very unlike the weak effect of QX-314 observed in pronase-treated axons (Fig. 8A). After repeated depolarizations, the current kinetics were also altered, as if the time-dependent block

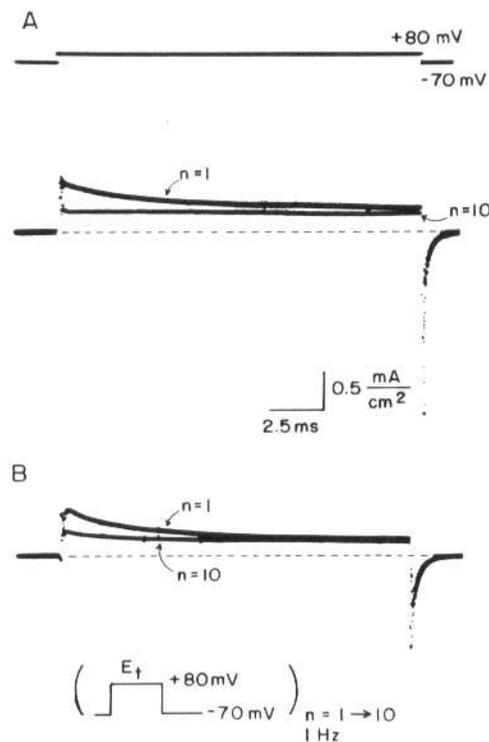


FIGURE 9. QX-314 and etidocaine modify the Na current kinetics in CT-treated axons during large, repetitive depolarizations. (A) In the presence of 1 mM QX-314, Na currents at +80 mV were measured before and after 10 pulses at the same potential. The peak current was blocked relatively quickly; in the second depolarization, >90% of the steady state block was achieved. In B, the same pulse protocol was used but the axon was perfused internally with 1 mM etidocaine.

were abolished (Fig. 9A). All of the remaining currents flow through Na channels, since they could be abolished by external tetrodotoxin at 100 nM. Assuming that most Na channels cannot enter an inactivated state in CT-treated axons, we propose that the binding of local anesthetics to open channels results directly in a time-dependent inhibition of Na currents and that this reaction increases during each depolarization and slowly decreases between depolarization

until it reaches steady state. Thus, the apparent time-dependent block disappears at steady state because no additional reactive (open) channels appear during the period between pulses and the anesthetic binding reaction has reached a true equilibrium. A similar explanation applies to the changes of current kinetics after repeated strong depolarizations of control axons (Fig. 7).

Another unexpected feature of the phasic block in CT-treated axons is its steeper voltage dependence (Fig. 8, *A* and *B*) for QX-314 and etidocaine. In addition, for both drugs, the maximum phasic block in CT-treated axons was slightly smaller than that in control axons but far greater than that in pronase-treated, QX-314-perfused axons. For example, QX-314 at 1 mM produced $\sim 66 \pm 9\%$ (SD, $n = 5$) of maximal block in CT-treated axons and $\sim 74 \pm 4\%$ ($n = 5$) block in the control axons. The reason for the slight increase in the limiting use-dependent block after CT treatment is unclear, although it is certain that in pronase-treated axons the phasic block of QX-314 is severely reduced. It is noteworthy that in CT-treated axons the remaining unblocked Na currents could be further blocked by QX-314 at higher concentrations. At internal 2 mM QX-314, an additional 42% of tonic block was achieved and 64% of the remaining current could be further blocked by repetitive pulses to +90 mV (1 Hz for 10 pulses); i.e., only $\sim 10\%$ of the original control Na current remained after repetitive pulses. This result demonstrates that most, if not all, of the Na channels in the CT-treated axons are sensitive to local anesthetics.

In addition to the increased voltage sensitivity of phasic block in CT-treated nerve, the functions were shifted on the potential axis in the hyperpolarized direction (Fig. 8). Consequently, at conditioning pulses of small amplitude, QX-314 and etidocaine actually produced more phasic block in CT-treated axons than in control axons (Fig. 10*A*). The rate of recovery from this phasic block (Fig. 10*B*), however, remained similar to the value ($t_{1/2} = 10\text{--}20$ s) reported for control axons (Cahalan, 1978). Table II summarizes the parameters used to generate the curves shown in Fig. 8, including the maximal phasic block (B_m), the midpoint potential (\bar{E}) that elicits the half-maximal block, and the slope (s) of the voltage-dependent block. Differences in these parameters do exist between the control and CT-treated axons and are discussed below.

In summary, the main results of this investigation are: (*a*) Quaternary and tertiary amine local anesthetics interact with open Na channels in normal perfused squid axons, resulting in a time-dependent inhibition of Na currents. (*b*) CT-modified channels are similarly inhibited by local anesthetics. Interactions with both normal and modified open channels appear to be voltage dependent, but at small depolarizations (E_m less than +40 mV), the time-dependent inhibition of control currents is smaller and slower than that of CT-treated currents. (*c*) After an open, noninactivating channel has been blocked by local anesthetics, recovery occurs slowly when the membrane is repolarized to a holding potential of -70 mV, and takes many seconds or even minutes to complete. This slow recovery accounts for the phasic block of these channels; inhibition initially increases more during each conditioning pulse than it decreases between pulses. No apparent differences in the kinetics of this reversal were found between control and CT-treated axons.

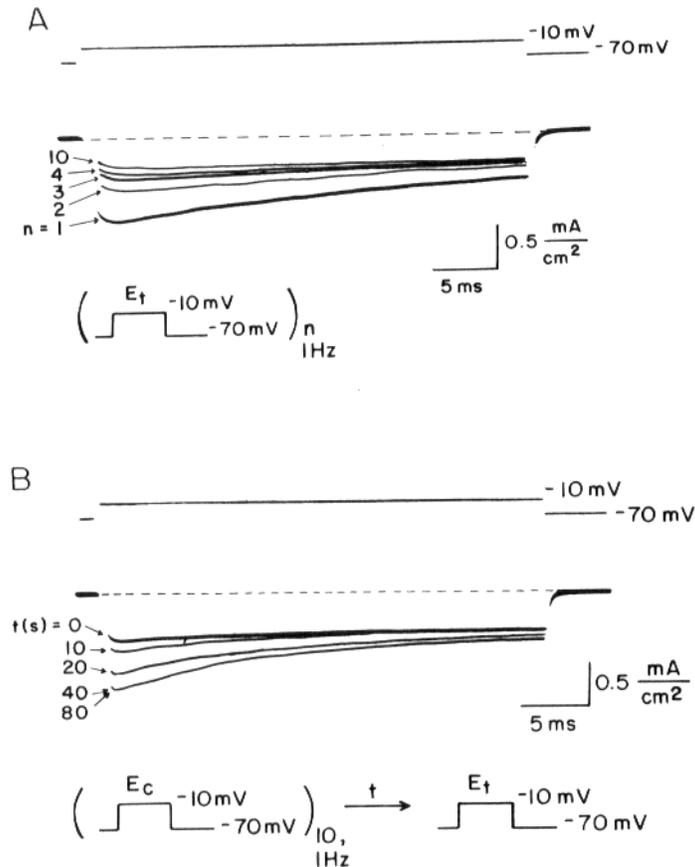


FIGURE 10. (A) The rate of onset of use-dependent block by QX-314 at -10 mV . The axon was perfused internally with 1 mM QX-314 for $>10\text{ min}$ before measurements began. (B) After a train of 10 repetitive depolarizations to -10 mV , the membrane was repolarized to -70 mV for increasing intervals (shown by numbers to the left of each current trace) and tested again at -10 mV to measure the Na current recovery. Each recovery time point was measured after a separate train of pulses so that no additional block occurred before the measurement.

DISCUSSION

Both CT and pronase treatment can inhibit Na channel inactivation in squid axons. Despite this similarity, in pronase-treated axons, QX-314 produces only a weak phasic block and elicits little time-dependent block, in agreement with previous reports (Cahalan, 1978; Yeh, 1978). However, in view of the new data on phasic block obtained from CT-treated axons, these previous observations should not be taken as evidence that Na channel inactivation is obligatory for the local anesthetic/channel interaction. Abolition of Na channel inactivation does not of itself lead to the reduction of phasic and time-dependent block.

One explanation for the decreased phasic effect of local anesthetics in pronase-treated axons is that the anesthetic affinity for an activated, open channel is reduced when some proteinaceous component of the Na channel is cleaved. Since CT has no proteolytic activity, such structural changes would not occur in the CT-treated axons.

Local Anesthetics Bind to Open Channels during Phasic Inhibition

Na current kinetics in the presence of local anesthetics are the complex result of channels that gate normally, those with drug-modified gating, and the actual drug binding and dissociation steps. A further complication arises at large depolarizations, where a significant proportion of Na channels do not inactivate. In this article, we treat this noninactivating population as a subgroup of normal channels, interconvertible with them, as proposed by Matteson and Armstrong (1982; see also Chandler and Meves, 1970). Local anesthetics similarly affect the

TABLE II
Characteristics of Use-dependent Block in Control and CT-treated Axons

	Etidocaine (1 mM)		QX-314 (1 mM)	
	Control	CT-treated	Control	CT-treated
B_{\max}	0.77	0.66	0.80	0.70
\bar{E} (mV)	-11	-26	-4	-30
s	15	8	18	10

These results were calculated from Fig. 8 using the equation

$$y = \frac{B_{\max}}{1 + \exp[(E_c - \bar{E})/s]} + (1 - B_{\max}),$$

where y is the relative I_{Na} at the test potential, B_{\max} is maximal use-dependent block, E_c is the conditioning membrane potential, \bar{E} is the potential that elicits half-maximal block, and s is an adjustable parameter that determines the maximum slope of the curve.

naturally occurring noninactivating channels and the CT-treated ones during a single depolarization.

Two experimental observations illustrate the role of the open channel in anesthetic block. First, in unmodified, strongly depolarized squid axons, the normal noninactivating currents are more inhibited than are the peak currents during a single pulse (Fig. 2), from which we conclude that the channels that remain open longer are blocked to a greater extent than those that inactivate. This difference is exaggerated in CT-modified axons, where even less of the current becomes inactivated during a single depolarization. There the steady state inhibition achieved by anesthetics during a sufficiently long pulse is greater for more positive conditioning potentials, which open more channels. Both this steady state level and the onset rate of inhibition increase with increasing anesthetic concentration. Therefore, the time-dependent decline in current during one pulse results from drug binding to open channels.

The second observation considers the differences between the steady state levels of phasic block in normal and CT-modified channels. Small repetitive depolarizations yield a relatively larger block of currents in CT-treated nerve

compared with untreated controls, while large depolarizations yield a relatively smaller block (Fig. 8). These differences can be explained by a combination of kinetic and thermodynamic factors. For small conditioning potentials, the voltage-dependent binding reaction is far from complete in a single pulse, for only a small fraction of all the channels will open at these potentials. However, since CT-treated channels remain open far longer than normal channels, the extent of anesthetic binding per pulse is greater in CT-treated channels and, since the rate of reversal of inhibition is not affected by CT treatment, the steady state inhibition achieved in the CT-treated axons is greater. In other words, the major potential dependence of steady state block under these conditions reflects the kinetic limitation imposed by the availability of open channels that can react rapidly with anesthetics. A similar alteration in the apparent voltage dependence of block by QX-314 was found by Cahalan and Almers (1979) when they partially replaced external Na^+ with impermeant Tris^+ , thereby accelerating the onset kinetics of phasic block of activated channels. Like the persistence of the open state produced by CT, the removal of extracellular Na^+ facilitated the drug-binding reaction.

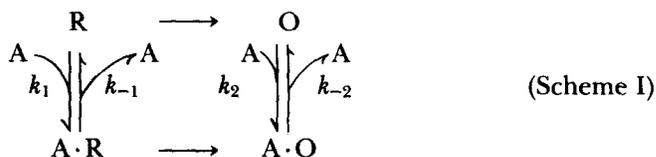
At larger conditioning potentials, a true equilibrium is approached since all channels are open with each pulse and relatively few of these inactivate in CT-treated and in control axons. In squid axons, the affinity of local anesthetics for inactivated channels appears to be slightly greater than for activated channels, for the inhibition is marginally increased when inactivation remains intact (Fig. 8). However, if anesthetic binding to inactivated channels far exceeded that to open channels, then the inhibition of control currents at large potentials would be much greater than that of CT-modified currents, a result that did not appear. Weak actions of CT on the anesthetic affinity of inactivating channels cannot be dismissed, however (i.e., an effect like that of pronase), even though the CT-modified open channels are inhibited in the time-dependent mode in much the same way as unmodified open channels.

Rate Constants of Anesthetic Binding Reactions

The binding of local anesthetics to the Na channel can be analyzed kinetically by examining the time course of the different aspects of inhibition of I_{Na} . This is possible because in most cases channel gating is clearly distinguishable from drug-binding reactions. Thus, at large depolarizations (E_m greater than +60 mV), the normal inactivation process is fast ($t_{1/2} < 0.7$ ms) compared with inhibition of open channels by either drug ($t_{1/2} > 2$ ms; Fig. 3). As a result, the kinetics of normal channel inactivation remain nearly unchanged or only slightly accelerated during a strong depolarization, but a slowly declining phase of I_{Na} becomes evident afterward as residual open channels are blocked.

The dissociation of anesthetic molecules from the Na channel after repolarization can be estimated by the rate of recovery from use-dependent block. This recovery process is slower ($t_{1/2} \approx 15$ s) than the normal gating functions of Na channels (also see Yeh and Tanguy, 1985), including the removal of slow inactivation (Narahashi, 1964; Adelman and Palti, 1969), and is probably limited by the dissociation of anesthetic molecules from the channels.

A simplified “modulated receptor” scheme accounts for the experimental observations in CT-treated nerves:



Here R represents the resting state of the Na channel; O is the open state and closed intermediates leading to it during activation; and A is a local anesthetic molecule. This scheme follows from the original model for the inhibition of K^+ channels by TEA^+ (Armstrong, 1969) and from the first scheme proposed for phasic block by quaternary lidocaine (Strichartz, 1973), as well as the aspect of Hille's (1977b) “modulated receptor” hypothesis that emphasizes the rapid, high-affinity binding of charged local anesthetics to the open rather than the resting or inactivated conformations of Na channels (cf. Schwarz et al., 1977).

Estimates of several of the rate constants of Scheme I are possible from our kinetic data. For 1 and 2 mM internal QX-314, the time constant and steady state value of time-dependent block in CT-treated axons at +120 mV permit the calculation of K_D (0.24 mM), the on rate constant ($k_2 = 2.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$), and the off rate constant ($k_{-2} = 68 \text{ s}^{-1}$) for the inhibition of open channels. This inhibition is about half as strong at -10 mV ($K_D = 0.43 \text{ mM}$), apparently because of an ~12-fold slowing of the apparent on rate ($k_2 = 2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and a 6-fold slowing of the off rate ($k_{-2} = 11 \text{ s}^{-1}$). However, at this potential, fewer of the total number of Na channels are available in any one pulse and the calculated bimolecular on rate constant will be underestimated by the fraction of channels that open.

These kinetic parameters are comparable to those calculated for phasic block of unmodified channels by brief depolarizations. The data for QX-314 in the normal squid axon conditioned to +10 mV yield $K_D = 0.75 \text{ mM}$, $k_2 = 2.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, and $k_{-2} = 18 \text{ s}^{-1}$ (estimated from Fig. 2 of Cahalan, 1978). For QX-314 in frog node (assuming an axoplasmic drug concentration of 0.4 mM), $K_D = 0.26 \text{ mM}$ with $k_2 = 1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_{-2} = 38 \text{ s}^{-1}$ at +75 mV (Strichartz, 1973). Schwarz et al. (1977) simulated the pH-dependent kinetics of use-dependent inhibition in frog muscle using rate constants for protonated lidocaine binding to open channels of $k_{\text{on}} = 2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{off}} = 0.5 \text{ s}^{-1}$ at -10 mV. In general, the rate constants for time-dependent I_{Na} inhibition in normal and CT-modified squid axons are close to the values estimated for use-dependent block of unmodified channels under similar conditions, which implies that the anesthetic affinity of open channels is altered little by CT.

Phasic block of currents by QX-314 occurs because of an incremental shift in channels from the R form to the nonconducting forms, A·R and A·O. Such shifts persist in the steady state because the recovery that occurs between the initial conditioning pulses is less extensive than the block that develops during those pulses. For Scheme I, the slower recovery must result from slow dissociations of drug from A·O or A·R, depending on which form exists in the

repolarized membrane. If drug-bound channels close normally (to $A \cdot R$ with $\tau < 1$ ms), they must then release their bound anesthetic slowly; if channels remain as $A \cdot O$ upon repolarization, then k_{-2} must be much smaller at -70 mV (~ 0.07 s $^{-1}$) than it is at -10 mV (11 s $^{-1}$) or $+120$ mV (68 s $^{-1}$).

Etidocaine also blocks I_{Na} in normal, CT-modified, and pronase-treated axons in both phasic and time-dependent modes (this article; Cahalan, 1978). The time-dependent block of open channels by etidocaine is as large or larger in pronase-treated axons ($k_{on} = 4.3 \times 10^6$ M $^{-1}$ s $^{-1}$; $k_{off} = 510$ s $^{-1}$; $K_D = 0.12$ mM; Cahalan, 1978) as in CT-treated axons ($k_{on} = 8.0 \times 10^5$ M $^{-1}$ s $^{-1}$; $k_{off} = 430$ s $^{-1}$, $K_D = 0.54$ mM; Fig. 6 of this article). As Cahalan (1978) noted previously, the sensitivity to pronase digestion of the etidocaine inhibition is modest, in contradistinction to the readily proteolyzed blocking activity by QX-314. Being a tertiary amine, etidocaine exists in equilibrium between protonated and neutral forms, either of which can bind to open channels via hydrophilic and hydrophobic pathways, respectively (Hille, 1977b; Schwarz et al., 1977). The primary access route of etidocaine may well be the hydrophobic route, for its pK_a at 10°C is ~ 7.4 , leaving it unprotonated half the time, and the hydrophobicity (octanol:buffer partition coefficient at 25°C) of the neutral species is 10^4 times that of the protonated species (Sanchez, V., G. R. Arthur, and G. R. Strichartz, unpublished measurements). Binding to open channels via the hydrophobic pathway thus appears to be pronase resistant, and phasic block by etidocaine probably results not so much from a restricted drug exit as from tighter binding to activated forms of the channel.

Role of Activation and the Origins of Voltage-dependent Inhibition

Gating of Na channels regulates local anesthetic action. The maximum phasic block by quaternary compounds is achieved by repeated brief depolarizations, although long pulses potentiate the block by lidocaine (Bean et al., 1983). The rates of onset and of recovery from phasic inhibition by tertiary and quaternary drugs are proportional to the probability of Na channels being open (Strichartz, 1973; Cahalan, 1978; Strichartz and Wang, 1986). This conclusion was also reached by Yeh and Tanguy (1985), who observed that the recovery from use-dependent block in squid is slower in hyperpolarized axons, even though normal inactivation is removed faster at more negative membrane potentials. Rather than indicating a controlling influence of inactivation on recovery, these results are consistent with dissociation of charged anesthetic molecules via activated channels, and thus the drug dissociation is decreasingly probable at more negative membrane potentials.

In agreement with the present report, the noninactivating component of Na currents in the CT-treated frog node of Ranvier is also sensitive to all species of local anesthetics: uncharged drugs (benzocaine; Ulbricht and Stoye-Herzog, 1984), tertiary amines (GEA-968; Strichartz and Wang, 1986), and quaternary amines (QX-314; Shepley et al., 1983), as well as antiarrhythmic compounds (*N*-propyl ajmaline and KC3791; Zaborovskaya and Khodorov, 1984). Therefore, it appears generally true that rapid inactivation gating is not required for phasic actions of local anesthetics on Na channels.

Despite the apparent voltage dependence of the rate constants, we do not believe that the membrane potential contributes directly to the free energy of the anesthetic binding reaction, for the following reasons. The Na conductance remaining at large depolarizations ($1 - B_{max}$; Fig. 7 and Table II) arises from unblocked channels that activate normally and can be inhibited further, with the same K_D , by higher anesthetic concentrations. If the drug-binding reaction per se were directly affected by voltage, and the K_D therefore decreased continuously with increased depolarization, then these channels would be blocked at sufficiently positive potentials (Strichartz, 1973; Cahalan, 1978; Cahalan and Almers, 1979). Instead, the actual voltage dependence of block saturates, which is consistent with the voltage-dependent transition of the channel among various forms having different affinities for local anesthetics.

If open channels are the only substrates for the increased binding of anesthetics in CT-treated axons, then the probability of block at equilibrium should be proportional to the open channel probability. The parameters that characterize the voltage dependence of block in control and CT-treated axons are listed in Table II. The comparable parameters for Na activation in CT-treated axons, $\bar{E} = -42$ to -45 mV and $s = 5-6$ mV (G. K. Wang et al., 1985), have a more negative midpoint potential and a steeper slope than those for anesthetic inhibition. However, these data are from experiments conducted in normal seawater containing a high concentration of Na ions, which appear to antagonize the phasic inhibition by quaternary local anesthetics (Cahalan and Almers, 1979). In solutions of lower external Na^+ , the phasic block in unmodified axons steepens and shifts to the left. This shift is equivalent to a change in the free energy of binding of the anesthetic; the drug binds more tightly, with a lower effective K_D , when there are fewer competing Na ions. We presume that the drug block in reduced Na^+ is relatively free of any ion competition that may itself be voltage dependent (Cahalan and Almers, 1979; Cahalan et al., 1980), and that the new voltage dependence more faithfully represents that of the anesthetic/channel interaction. The shift in voltage dependence upon Na^+ replacement, ~ 20 mV negative, when added to our values for the voltage-dependent block in CT-treated axons (Table II), yields midpoint potentials of -46 to -50 mV, which is close to the range of E for channel activation, -42 to -45 mV. Thus, the true voltage dependence of both the phasic (and time-dependent) inhibitions by local anesthetics may arise exclusively from the conformational changes that attend channel activation.

The Role of Channel Inactivation

The inactivated state of Na channels also appears to have a high equilibrium affinity for local anesthetics, but seems to bind and dissociate these drugs at lower rates than do the open channels. Manipulations of the inactivated state through control of membrane potential can alter the potency of local anesthetics by two modes. First, small depolarizations of long duration will increase the channel's affinity for anesthetics, apparently by converting channels to an inactivated state (Hille 1977a, b; Bean et al., 1983). Small hyperpolarizing pulses reversibly relieve the inhibition by neutral, tertiary (Courtney, 1975; Hille,

1977b), and quaternary amine (Schwarz et al., 1977) anesthetics, although the latter reaction occurs relatively slowly, requiring 8–12 pulses to complete half the reaction. The second role of inactivation is to govern the rapid reaction of anesthetics with open channels. Thus, inactivation produced by a small depolarizing prepulse (preceding a large conditioning pulse, to +75 mV) slows the rapid phasic block in frog nerve by QX-314 (which otherwise has a “half-time” of one to two pulses), as well as the reversal of such block that is mediated by smaller depolarizations (Strichartz, 1973). Which mode of inactivation is more important depends on the particular drug and on the pattern of applied potential.

Inactivation may slow rapid anesthetic binding by reducing the probability of open channels or by directly hindering access to the anesthetic-binding site. Recently, Starmer et al. (1984) suggested that restriction of the drug from the local anesthetic-binding site by the activation gate could by itself account for use-dependent block. In their model, the equilibrium affinity of all channel states for local anesthetics is the same, and the true K_D for QX-314 in squid axons is $\sim 1.3 \times 10^{-9}$ M. A dose of $>1,000 K_D$ is required for observed block because the binding site is only transiently accessible. Our results do not support this “guarded receptor” hypothesis. Even when the binding sites of the Na channels are kept available during strong depolarization, as in CT-treated axons, the binding affinity of different states does not differ significantly from that of their counterparts in the untreated axons. The apparent K_D for QX-314 at +120 mV is ~ 0.24 mM in CT-treated axons; in resting CT-treated axons, the K_D must increase to 1.7 mM to account for tonic inhibition. The values for tonic and steady state use-dependent inhibition in normal (“guarded”) channels are quite similar. The true K_D for QX-314 is unlikely to be in the nanomolar range; otherwise, all the currents should be abolished by 1 mM QX-314 during phasic block of CT-treated axons.

Note added in proof: The models of Starmer et al. (1986. *Biophysical Journal*. 49:913–920), published since the present work was submitted, estimate rate and equilibrium dissociation constants for “trapped” channel block by a quaternary amine anesthetic that are quite comparable to the values we report here.

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