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# Interaction of Spin-Labeled Local Anesthetics with the Sodium Channel of Squid Axon Membranes

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**Summary.** The effects of spin-labeled local anesthetics on sodium currents of internally perfused squid axons were studied using the voltage-clamp technique. Internal application (10 μM) of the most potent spin-labeled local anesthetic used in this study produced a small initial block of sodium currents. However, after sixty repetitive pulses (to +80 mV) given at 1 Hz, the sodium currents were drastically reduced. In addition to this frequency-dependent phenomenon, the anesthetic effect on the sodium currents was also sensitive to the voltage of the pulses. Both the frequency- and voltage-dependent properties remained intact after removal of sodium inactivation with pronase. The recovery of sodium currents from this frequency-dependent anesthetic effect followed a single exponential curve with a surprisingly long time constant of about 10 min. Such a long recovery time, which is longer than any known sodium inactivation process, led us to suggest that the recovery process represents the dissociation of drug molecules from their binding sites. We have also found that increasing hydrophobic character of the homologues series of spin-labeled local anesthetics enhances the frequency- and voltage-dependent block of sodium currents. This effect strongly suggests that hydrophobic interaction is an integral component of the binding site. These probes with their selective effects on the sodium currents, are expected to be highly useful in studying the molecular structure of the sodium channels.

**Key words** local anesthetics · voltage-clamp · squid axon · sodium channels · spin label · frequency dependence

## Introduction

The spin-labeling technique is an important method for studying biological and bilayer membranes; more specifically, spin-labeled local anesthetics are useful tools in probing the mechanism of nerve membrane excitation. In order to utilize spin-labeled local anesthetics to study mechanisms of excitation, it is necessary to know their effects on the flow of ionic currents in nerve membranes. We have previously demonstrated the binding properties (Giotta, Gargiulo & Wang, 1973; Giotta, Chan & Wang, 1974) and the kinetics of chemical blockade (Yeh, Takeno, Rosen & Narahashi, 1975) for some spin-labeled local anesthetics. In this study we have investigated the ionic mechanism of action of spin-labeled local anesthetics; our study included both tertiary amines and one of the quaternary derivatives. The quaternary derivative at very low concentrations exhibited a very strong frequency- and voltage-dependent block of Na channels. Emphasis in this paper is placed on these frequency- and voltage-dependent local anesthetic effects.

## Materials and Methods

Giant axons isolated from squids, *Loligo pealei,* obtained at the Marine Biological Laboratory, Woods Hole, Mass., were internally perfused by the roller method originally developed by Baker, Hodgkin and Shaw (1961) and modified by Narahashi and Anderson (1967). The axon, mounted in a Plexiglas chamber, was perfused internally with standard internal solution (SIS) and externally with artificial seawater (ASW). The axon was voltage clamped using the conventional axial wire technique (Wu & Narahashi, 1973), but with improved voltage control by using double electrode assemblies closely positioned on both sides of the axon. Each of these electrode assemblies consists of three platinum-plated electrodes, with the middle one measuring membrane current and the two exterior ones electrically guarding against nonradial current flow into the central plate. Leakage currents and most of the capacitive currents were electronically subtracted from the records by assuming linear current voltage relationships. Approximately two-thirds of the series resistance was compensated by a feedback circuit. The membrane was usually held at −80 mV except during holding potential experiments. Membrane potential measurements were corrected for junction potential. In all experiments temperature was maintained at 10±0.5 °C as measured by a thermocouple mounted as close to the axon as possible in the central current-measuring electrode region. Normal ASW had the following composition (mM): $Na^+$, 450; $K^+$, 10; $Ca^+$, 50; $Cl^-$, 575; HEPES buffer, 5. The final pH was adjusted with NaOH or HCl to 8.0, and the osmolarity was about 980 mosmol. The SIS had the following composition (mM): $K^+$, 350; $Na^+$, 50; glutamate$^-$, 320; $F^-$, 50; phosphate buffer, 15; sucrose, 333. The pH was adjusted to 7.3 with KOH or HCl. The osmolarity of SIS was approximately 1020 mosmol. In order to suppress the potassium current, tetraethylammonium (TEA) fluoride was added to SIS to make a final TEA concentration of 20 mM.

The chemical structure of the spin-labeled local anesthetics used in this study is shown in Fig. 1. These compounds, 2-[N-methyl-N-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl *p*-alkoxyben-

0022-2631/82/0066-0227 $01.40

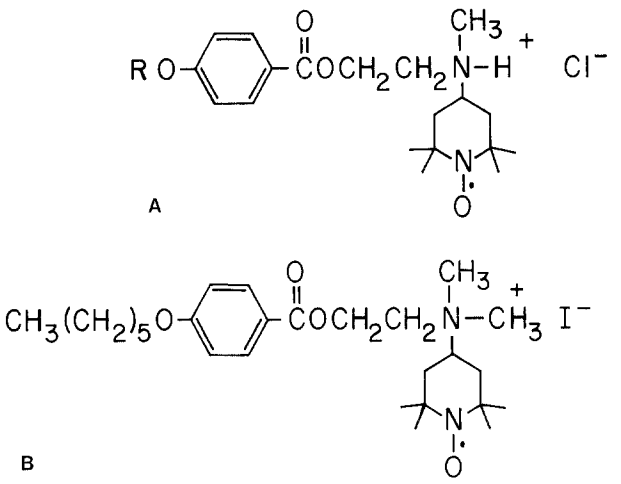


**Fig. 1.** Chemical structures of spin-labeled local anesthetics of the tertiary amine derivatives (shown in *A*) are abbreviated C2SL, C4SL, and C6SL, respectively, for spin-labeled derivatives containing *R* groups of $CH_3CH_2-$, $CH_3(CH_2)_3-$, and $CH_3(CH_2)_5-$. The quaternary derivatives with $R=CH_3(CH_2)_5-$ (shown in *B*) is abbreviated as C6SLMEI

zoates, are abbreviated as C2SL, C4SL, and C6SL according to the number of carbons in the alkoxyl groups; the quaternary derivative of C6SL is abbreviated as C6SLMEI. The synthesis of these compounds were previously reported (Gargiulo, Giotta & Wang, 1973).

Membrane currents, recorded on film, were analyzed with the help of a digitizer linked to a programmable calculator (HP 986A digitizer and HP 9821 calculator, Hewlett Packard, Palo Alto, Calif.).

## Results

### *Frequency-Dependent Suppression of Action Potential and Frequency-Dependent Inhibition of Sodium Currents*

As illustrated in Fig. 2*A*, the membrane action potential in a quiescent axon that was internally perfused with 10 μM C6SLMEI appeared normal initially but gradually decreased in the rate of rise and amplitude and finally was blocked during trains of stimuli given at 1 Hz. Figure 2*B* shows that under voltage clamped conditions, the inward sodium current associated with step depolarization to 0 mV was only slightly suppressed during the first pulse (trace labeled *1*). The difference between this current and the control current represents the initial resting block. The Na current was progressively suppressed by repetitive pulsing to the same potential at 1 Hz. After 60 pulses, less than 10% of the original inward Na current was observed (trace labeled *60*). This additional block of the currents over and above the initial block is defined as frequency- (or use-) dependent block. This frequency-dependent inhibition of Na currents is sufficient to account for the frequency-dependent suppression of action potentials described in Fig. 2*A*.

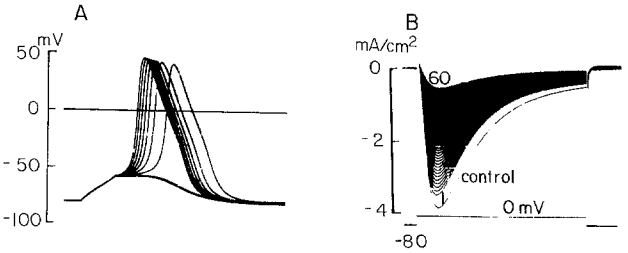


**Fig. 2.** Frequency-dependent block of membrane action potential (*A*) and frequency-dependent suppression of sodium current (*B*) by an internal application of 10 μM C6SLMEI. Action potentials and sodium currents were both elicited at a frequency of 1 Hz

### *C6SLMEI Selectively Blocks Na Channel*

C6SLMEI at internal concentration of 10 μM exerted an inhibitory effect on Na channels as a result of the accumulated block produced by repetitive pulsing. Figure 3*B* shows the initial resting block of Na and K currents by 10 μM C6SLMEI. Both inward and outward going Na currents were only slightly suppressed in this quiescent axon. However, following trains of pulses to +80 mV given at 1 Hz for 60 times, the Na currents were almost completely abolished (Fig. 3*C*). Potassium currents were not affected at −20 mV and slightly suppressed at +80 mV, suggesting that there is a voltage-dependent block of K channels. A family of membrane currents taken after this conditioned pulse is illustrated in Fig. 3*C*; when this is compared to control currents in Fig. 3*A*, it is very clear that the predominant effect of C6SLMEI is on Na currents. The current-voltage relationship shown in Fig. 4 further illustrates the selective effect on Na channels. This paper was aimed at characterizing the interaction of spin-labeled probes with Na channels. The small effect on K channels is voltage dependent, but was not investigated further in this paper.

### *Sodium Inactivation and C6SLMEI Block of Na Currents*

The fast Na inactivation (*h*) process has been found to play an important role in local anesthetic blocking action on Na channels (Strichartz, 1973; Courtney, 1975; Hille, 1977; Cahalan, 1978; Yeh & Wu, 1978; Yeh, 1979). Its role in C6SLMEI block of Na currents was investigated using pronase-treated axons in which the fast Na inactivation was removed. The effect of the removal of Na inactivation upon blockage of Na channels by C6SLMEI is discussed in terms of its frequency dependence and its voltage dependence.

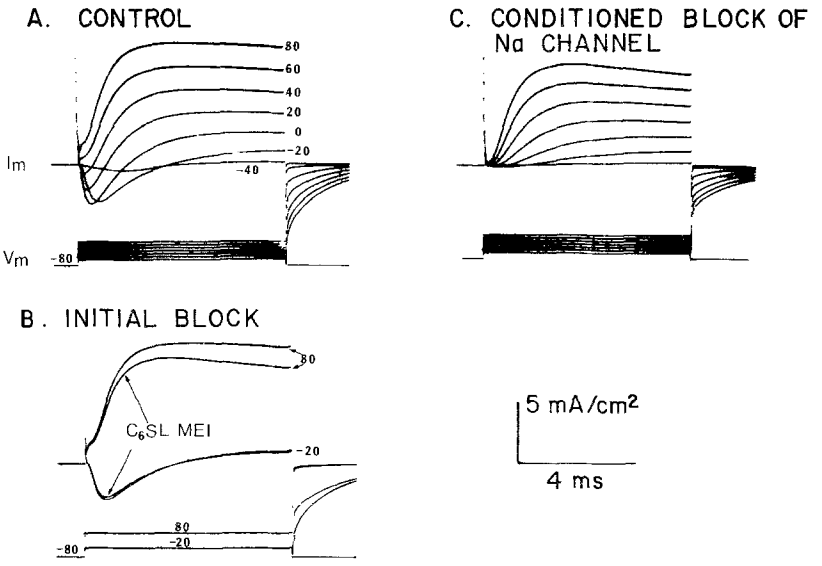


**Fig. 3.** Families of membrane ionic currents ($I_m$) in response to step depolarization potentials ($V_m$) before (*A*) and during the presence of 10 μM C6SLMEI (*B* and *C*). The $V_m$ values are labeled near the corresponding membrane current traces in *A* and *B*. The $V_m$ values in *C* are the same as those in *A* and are not labeled. As shown in *B*, in a quiescent axon, 10 μM C6SLMEI produced only a small initial block of sodium and potassium currents. In *C*, the axon had been conditioned prior to obtaining the membrane currents, the sodium currents were selectively blocked by C6SLMEI. The conditioning potentials consisted of trains of 60-step depolarizations to +80 mV each 8 msec in duration and given at 1 Hz

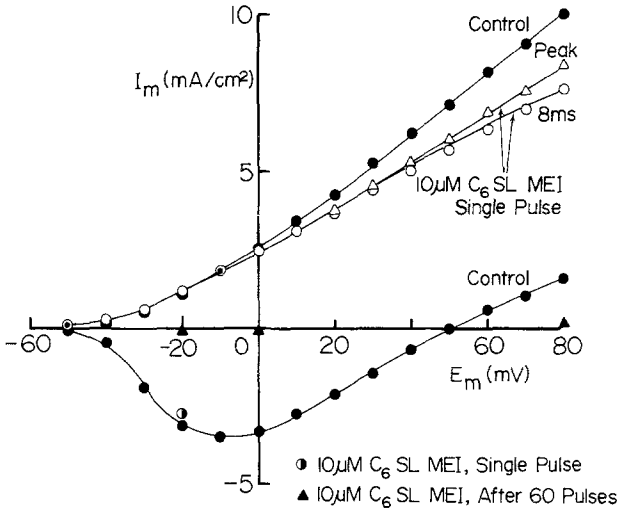


**Fig. 4.** The current-voltage relations for sodium and potassium currents were affected differently by 10 μM C6SLMEI. The drug produced a small decrease in Na current (0 at −20 mV) but eliminated it almost completely (▲) following 60 pulses to +80 mV given as described in Fig. 3*C*. The potassium currents, either measured at the peak or isochronically at 8 msec, were slightly suppressed when $E_m$ was more positive than 0 mV

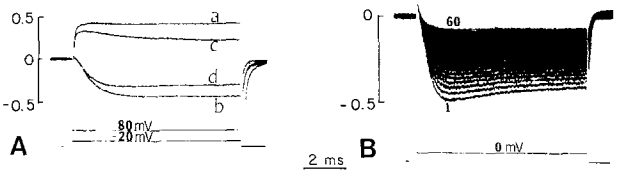


**Fig. 5.** In a pronase-treated axon, 10 μM C6SLMEI produced a time-dependent block of Na current (*A*) and a frequency-dependent block of Na currents (*B*). The pulses were given at a frequency of 1 Hz in *B*

*Frequency Dependent Block.* Following the removal of Na inactivation by pronase treatment, Na currents rose normally, but failed to decrease during 8 msec depolarization step as illustrated in Fig. 5*A*. The current traces *a* and *b* (Fig. 5*A*) representing Na currents during step depolarizations to +80 and −20 mV, respectively, were suppressed to the traces *c* and *d*, respectively, following drug application. C6SLMEI exhibited a time-dependent block of Na currents, which was especially discernible at higher depolarizations (the upper trace *c* in Fig. 5*A*). This time-dependent decay of Na current has been interpreted as the reflection of molecular interaction of drug molecules with open channels (Shapiro, 1977; Yeh & Narahashi, 1977; Almers, 1979*a*, *b*; Yeh, 1979). The frequency-dependent block was observed upon repetitive pulsing to 0 mV as illustrated in Fig. 5*B*. The Na currents in the presence of drug is gradually decreased beginning with trace *1*, and ending with trace *60* as shown in Fig. 5*B*, as seen in axons with intact inactivation (Fig. 2*B*). However, the onset rate of frequency-dependent block of Na currents was accelerated following the removal of the inactivation process. The time constants (in terms of number of pulses as 1 Hz) for the onset of the frequency-dependent block (induced by 10 μM C6SLMEI) were 26±2.0 pulses and 10±2.4 pulses (mean±SD, $n=3$), respectively, before and after pronase treatment. The fact that most of the frequency-dependent block remains intact after removal of sodium inactivation suggests that sodium inactivation does not play as important a role in this case as that involving procaine and QX-314 where the frequency-dependent block was almost completely abolished following the removal of the inactivation gate (Cahalan, 1978; Yeh & Wu, 1978).

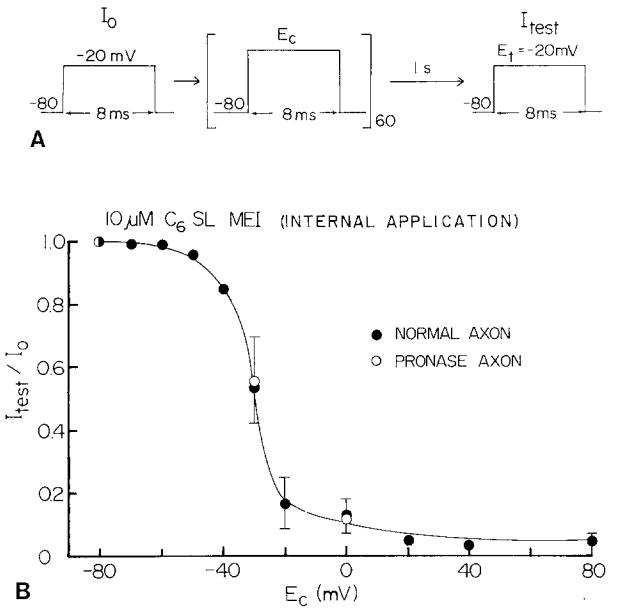


**Fig. 6.** Pronase treatment does not affect a voltage dependence of the frequency-dependent block of sodium current. To remove Na inactivation the axon was pretreated with 0.2 mg/ml pronase for 10 to 15 min. (*A*): The protocol used to evaluate the voltage dependence of the frequency-dependent block of sodium current is illustrated. The peak Na currents associated with test pulse following the conditioning protocol ($I_{test}$) were normalized to the peak Na current associated with the initial pulse ($I_o$). (*B*): The normalized values ($I_{test}/I_o$) were plotted as a function of the conditioning potentials ($E_c$)

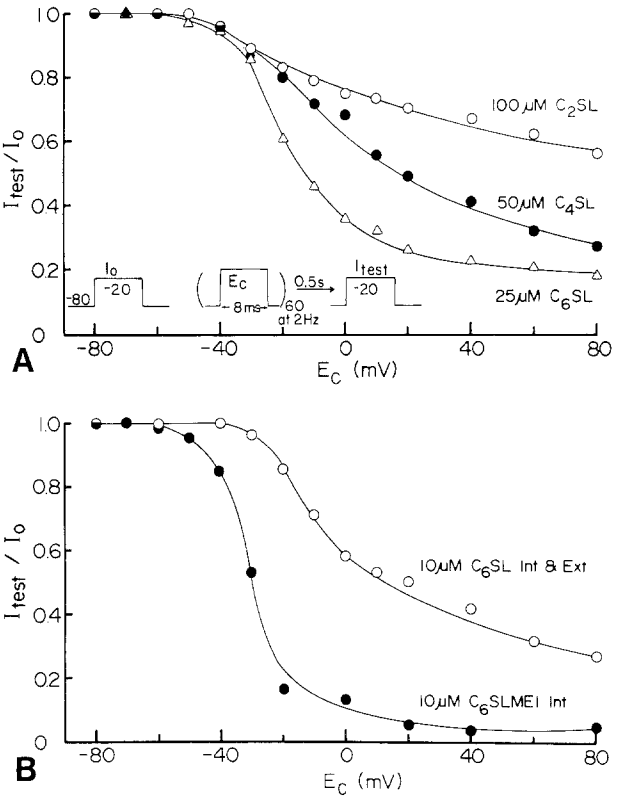


**Fig. 7.** The spin-labeled local anesthetics differ in voltage dependence as their structures are changed. The dose was chosen to produce an equal decrease in Na current (resting block) in a quiescent axon. The protocol was illustrated in the inset of *A*. Lengthening the alkoxyl chain or quaternization of the nitrogen enhanced the steepness of voltage dependence

*Voltage-Dependent Block.* The degree and extent of frequency-dependent block of Na current depend on the amplitude of conditioning potentials. This voltage dependence can be estimated by a protocol as illustrated in Fig. 6*A*. The peak Na currents associated with the test pulse ($I_{test}$ to $-20$ mV was applied after conditioning potentials) were normalized to the current associated with the initial pulse ($I_o$ at $-20$ mV was applied after 5 min rest at $-80$ mV); the normalized values were plotted in Fig. 6*B*. The blockage was enhanced by conditioning pulses at potentials where additional channels were opening, but the blockage was saturated at potentials where all available channels were fully open. The conductance-dependent block thus manifested itself as a steeply voltage-dependent block.

### *The Effect of Anesthetic Structure on Voltage-Dependent Block of Na Channels*

The voltage dependence of Na channel block depends on the structure of spin-labeled local anesthetics. Lengthening the alkoxyl chain substitution from C2 to C6 greatly enhances the voltage-dependent block. Figure 7*A* illustrates that voltage dependence in the presence of 25 μM C6SL is more pronounced than that exhibited by 50 μM C4SL, which is in turn more pronounced than that seen in the presence of 100 μM C2SL. Quaternization of tertiary amine enhanced the voltage dependence, illustrated in Fig. 7*B*. The tertiary compound is permeant to the membrane while the permanently charged quaternary compound is not; therefore it is possible to attribute their different potencies to diffusional loss of C6SL across the membrane. To avoid such unequal distribution of drug molecules, the drug effects were compared at equilibrium. In the case of tertiary derivative C6SL, the drug was applied both internally and externally to the axon to achieve the equilibrium concentration. Quaternary amine derivative C6SLMEI was applied internally since external application did not enhance the blocking action. Figure 7*B* shows that at equilibrium, and with both drugs at 10 μM concentrations, C6SLMEI block is more pronounced than C6SL block; the difference is most dramatic at lower depolarization potentials between $-30$ and 0 mV. In the

case of C6SLMEI block, the voltage dependence seemed to saturate at relatively low potentials and the difference between +80 and 0 mV is rather small. In the case of C6SL block, the degree of block does not seem to saturate even at +80 mV. Since the effect was compared at the same concentration of drug at equilibrium, the profound difference between tertiary and quaternary derivatives is most likely reflecting the true difference in drug binding to the site of action.

*Recovery from Frequency-Dependent Block*

The recovery rate from local anesthetic-induced frequency-dependent block of the Na channels varies. This variation may represent different rates of dissociation of local anesthetics from their binding sites. The recovery from frequency-dependent block of Na channels in the presence of spin-labeled local anesthetics was slow. Two of them, C6SL and C6SLMEI, were studied in great detail and reported here.

Recovery from C6SLMEI block was two and a half times slower than recovery from C6SL block. The kinetics of recovery were measured using the pulse protocol shown in the inset of Fig. 8. Sodium channels were first blocked to a steady-state level by pulsing 60 times at 2 Hz to +80 mV. Recovery from this conditioned block was monitored by a constant test pulse to 0 mV applied at various intervals after the last conditioning pulse. The sodium currents ($I_t$) associated with the test pulses were normalized to the inital value of the sodium current ($I_\infty$) *obtained* 30 min following the last conditioning pulses. These normalized data were plotted as a function of pulse interval (*see* Fig. 8).

Recovery of Na channels from frequency-dependent block followed a single exponential time course. The time constants were $4.1 \pm 2.6$ (mean $\pm$ SD, $n=6$) and $10.3 \pm 3.3$ min (mean $\pm$ SD, $n=6$), respectively, for C6SL and C6SLMEI. Since the time constants of recovery from C6SL and C6SLMEI blocks are both very slow, the dissipation of the accumulated block during the interpulse interval at rest is expected to be minimal. Therefore, the main difference in potency between the two compounds is probably attributed to their difference in the kinetics of interaction with Na channels during step-depolarizing pulses.

*Effect on Slow Inactivation*

As shown in Fig. 7*B*, repetitive step depolarization to −50 mV of 8 msec pulse duration and at 2 Hz was not effective in inducing the frequency-dependent block by C6SL, whereas a prolonged depolarization to −50 mV lasting several minutes effectively blocked

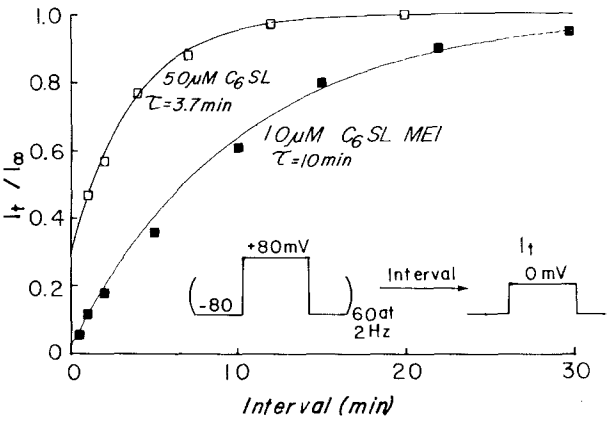


**Fig. 8.** Time course of recovery from the frequency-dependent block of Na currents by C6SL and C6SLMEI. The protocol is illustrated in the inset. The data can be fit by a single exponential function of the form 1-(1-$A$)exp($-t/\tau$), where the value of $A$ are 0.4 and 0.02 for C6SL and C6SLMEI, respectively, and the time constants ($\tau$) are 3.7 and 10 min, respectively, for C6SL and C6SLMEI. In the case of C6SL, an appreciable fraction of Na current (about 40%) was recovered with time constant of 3 to 4 msec and saturated 30 msec after the lasting conditioning pulse, which probably represented a population of channel free of drug molecule

all Na channels. This latter effect was studied using a protocol in which the membrane potential is held at different potentials for 5 min before a pair of prepulses and test pulses were applied (*see* the inset in Fig. 9*B*). The Na currents thus obtained are plotted, in a value relative to the control, as a function of the 5-min holding potential in Fig. 9*A*. The same data are plotted in Fig. 9*B* in a value normalized to the respective maximum Na current. C6SL was found to suppress Na current in a manner dependent on the holding potential. The block was enhanced by holding the membrane at more depolarized levels and relieved to some extent by the hyperpolarizing potential.

The ratio of Na current to its own maximum value plotted as a function of holding potential was designated as the slow inactivation curve ($S_\infty$) (*see* Fig. 9*B*). C6SL shifted the $S_\infty$ curve to the hyperpolarizing direction and increased the steepness of the slope. Simultaneous application of C6SL from both sides of the membrane produced a greater blocking effect on the Na current and a greater shift in the $S_\infty$ curve than internal application only. This difference probably arose from the fact that C6SL is a tertiary amine and the neutral form can quickly diffuse out of the axon, and becomes unavailable to the internal sites. C6SLMEI produced a similar shift, but only from internal application. This shift in $S_\infty$ curve to the hyperpolarizing direction would be manifested itself as a tonic block if the axon were held at its normal holding potential of −80 mV.

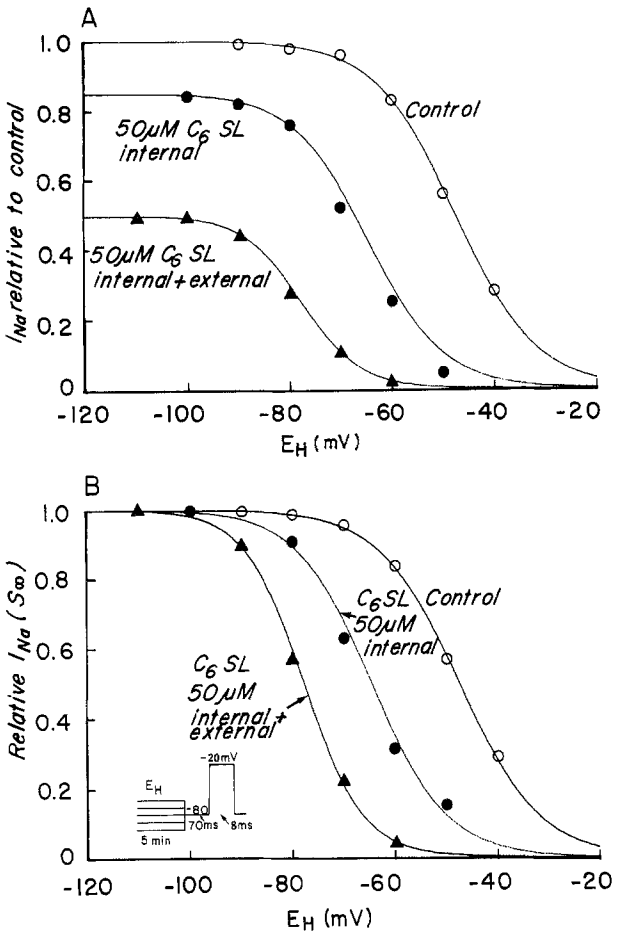


**Fig. 9.** The C6SL effects on the resting block is dependent on the holding potential (*A*). The slow Na inactivation curve is shifted to the hyperpolarizing direction (*B*). The pulse protocol used to measure the resting block is shown in the inset of *B*. (*A*): The sodium currents associated with test pulse to −20 mV were normalized to maximum Na currents of the control. (*B*): Na currents associated with test pulse to −20 mV were normalized to its own maximum value in each case, and these normalized values were plotted as a function of holding potentials ($E_H$). This is called a slow inactivation curve ($S_\infty$). *Internally and externally simultaneous application of C6SL was more effective than internal application of C6SL in producing a shift in* $S_\infty$

## Discussion

A slow recovery from blockage upon repolarization predicts that with repetitive stimulation the persistence of blocker in the channel will lead to cumulative block. This phenomenon has been studied extensively with many local anesthetics (Strichartz, 1973; Courtney, 1975, 1980; Hille, 1977; Schwartz, Palade & Hille, 1977; Cahalan, 1978; Kendig, Courtney & Cohen, 1979; Yeh, 1979) and is termed frequency- or use-dependent block. Spin-labeled local anesthetics studied here, C2, C4, C6 derivatives, all share the features of frequency-dependent block of Na channels exhibited by other local anesthetics as previously reported.

The modulated receptor hypothesis (Hille, 1977) states that drug molecules have different affinities for the blocking site, depending on the gating state of the channel. This gate-dependent binding can thus manifest itself as frequency- and voltage-dependent block of ionic channels. Removal of the Na inactivation by pronase would allow more channels to be opened for drug binding during step depolarizations. Therefore, the observation that pronase treatment accelerated the onset of frequency-dependent block is consistent with the interpretation that C6SLMEI molecules primarily interact with open channels. The fact that the steady-state voltage-dependent block is not changed by pronase treatment suggests that pronase treatment does not affect the drug binding site but, rather, increases the availability of Na channels for opening.

Among these compounds, C6SLMEI is most interesting in two respects. First, the frequency- and voltage-dependent block of Na channels does not depend on the intactness of Na inactivation mechanism, and secondly, the time course of recovery from this block is extremely slow with time constant being longer than 10 min. There are no known physiological processes of Na inactivation which have a time constant of longer than 10 min. We postulate that this recovery process probably represents the dissociation of drug molecules from their binding sites at the Na channels. The finding that lengthening the alkoxyl chain at the para-position of the benzene ring enhances the frequency- and voltage-dependent block strongly suggests that hydrophobic interaction is an integral component of the binding site in the Na channel. Similar conclusions regarding the hydrophobic interactions has been drawn from other studies on Na channels with guanidine derivatives and tetraethylammonium derivatives (Kirsch, Yeh, Farley & Narahashi, 1980; Rojas & Rudy, 1976).

It has been proposed that the molecular size of local anesthetics is an important determinant of the recovery rate from frequency-dependent block (Courtney, 1980). However, in a homologous series of local anesthetics such as C6SL, the increase in molecular weight is accompanied by an increase in hydrophobicity of the anesthetic. Gargiulo et al. (1973), using surface anesthesia in the guinea pig cornea, has observed an increase in duration of anesthesia as the hydrophobic chains are lengthened. They suggested that duration of anesthesia of these compounds is correlated to their ability to dissolve in the hydrocarbon region of the membrane as shown by the partition order: C6SL > C4SL > C2SL (Giotta et al., 1973). The precise role of the hydrophobic ef-

fect on the interaction between local anesthetics and various membrane components (e.g., channel protein *vs.* membrane lipids) remains to be determined.

Due to its rather selective effect on Na channels, C6SLMEI is a useful probe for studying properties of Na channels *in situ*. ESR signals, taken before and after the conditioned block in the presence of C6SLMEI may reveal differences which represent selective binding of local anesthetic molecules to Na channels. Similar approaches using these anesthetic probes have been successfully demonstrated in model systems (Wang, Earnest & Chan, 1980).

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