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Ionic Mechanism of Action of a Spin-Labeled Local Anesthetic on Squid Axon Membranes

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Summary. The ionic mechanism of action of a spin-labeled local anesthetic (SLA), 2-[N-methyl-N-(2,2,6,6-tetramethylpiperidinooxyl)]-ethyl 4-ethoxylbenzoate, was studied by means of voltage clamp technique with squid giant axons in comparison with the parent compound without spin label moiety, 2-(N,N-dimethyl)ethyl 4-ethoxylbenzoate (GS-01). Like other local anesthetics, they suppressed both sodium and potassium conductance increases. However, three remarkable differences have been noted between SLA and GS-01: (1) SLA is more effective than GS-01 in suppressing the sodium and potassium conductance increases; (2) SLA induces a potassium inactivation, whereas GS-01 is lacking this ability; (3) SLA has no effect on the time to peak sodium current, whereas GS-01 prolongs it. GS-01 resembles procaine with respect to (2) and (3) above. SLA will become a useful probe for the study of the molecular mechanism of local anesthetic action and of ionic channel function.

The ionic mechanism of action of local anesthetics has been a matter of extensive studies during the past 15 years (Shanes, Freygang, Grundfest & Amatniek, 1959; Taylor, 1959; Blaustein & Goldman, 1966; Narahashi, Moore & Poston, 1969; Narahashi, Frazier & Moore, 1972). Local anesthetics suppress both sodium and potassium conductance increases that occur during nerve excitation, and the inhibition of the former is directly responsible for conduction block. It has also unequivocally been demonstrated that tertiary amine local anesthetics act from inside of the axonal membrane in the cationic form after having penetrated through the membrane in the uncharged form (Frazier, Narahashi & Yamada, 1970; Narahashi, Frazier & Yamada, 1970; Narahashi & Frazier, 1971, 1975). However, the molecular mechanism of the interaction between the local anesthetic and the sodium or the potassium channel

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remains to be elucidated. Our continuing interest in local anesthetics has prompted us to seek a new approach to the problem.

In recent years, the use of nitroxides as free-radical probes in the study of biological membranes has provided some new information on the fluidity of the membrane (McConnell & McFarland, 1970; Jost, Waggoner & Griffith, 1971; McConnell, Wright & McFarland, 1972). Rosen (1974) and Gargiulo, Giotta & Wang (1973) have independently synthesized spin-labeled local anesthetics. Before undertaking detailed spin-label analyses for the molecular interaction of local anesthetics with nerve membranes, it is of paramount importance to understand the ionic mechanism of action of the spin-labeled local anesthetic as compared to the parent compound without the spin-labeled moiety. This was the objective of the present investigation. Our experiments have clearly shown that a spin-labeled local anesthetic acts differently from its parent, a nonspin-labeled local anesthetic, in two aspects; i.e., the spin-labeled local anesthetic induces a potassium inactivation which is not seen in the normal axon or the axon exposed to the nonspin-labeled local anesthetic, and is more potent than the nonspin-labeled one.

Materials and Methods

Giant axons from the squid *Loligo pealei*, available at the Marine Biological Laboratory, Woods Hole, Massachusetts, were used throughout the experiments. All the experiments were carried out at a temperature of 13–15 °C.

The isolated giant axon was cleaned of connective tissues and then nerve fibers, and mounted in a nerve chamber. It was continuously perfused externally with artificial seawater with or without test compound. The composition of artificial seawater is as follows (mM): 450 Na^+, 10 K^+, 50 Ca^{++}, 10 tris(hydroxymentyl)-aminomethane(Tris) and 575 Cl^-, at pH 8.0.

The axial wire voltage clamp technique used in the present study was essentially the same as that described previously (Wang, Narahashi & Scuba, 1972; Wu & Narahashi, 1973). The method of calculation of membrane conductances has been described previously (Yeh & Narahashi, 1974*a*). When potassium current underwent an inactivation under the influence of spin-labeled local anesthetic, both the peak value and the value at the end of a 7-msec pulse were measured, and the conductance at the peak current was referred to as the peak potassium conductance.

The method of internal perfusion was essentially the same as that described previously (Narahashi & Anderson, 1967). The standard internal solution contained (mM): 50 Na^+, 350 K^+, 50 F^-, 320 glutamate$^-$, 15 $H_2PO_4^-$ and 330 sucrose, at pH 7.3.

The local anesthetic (GS-01) and its spin-labeled analog (SLA) used in the present study were synthesized as described elsewhere (Rosen & Ehrenpreis, 1972; Rosen, 1974). They are 2-[N-Methyl-N-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl-4-ethoxylbenzoate and 2[N,N-dimethyl]ethyl-4-ethoxylbenzoate, respectively, and the chemical structures are shown in Fig. 1.

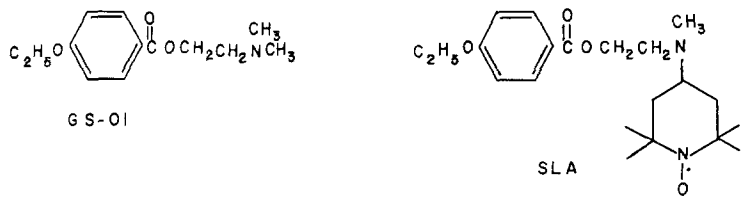

Fig. 1. Chemical structure of nonspin-labeled local anesthetic, 2-[N,N-dimethyl]ethyl-4-ethoxylbenzoate (GS-01) and spin-labeled analog, 2-[N-methyl-N-(2,2,6,6-tetramethylpiperinooxyl)]ethyl-4-ethoxylbenzoate (SLA)

Results

Effect on Membrane Currents

Spin-labeled local anesthetic inhibited both sodium and potassium currents associated with step depolarizations. In addition, it induced a potassium inactivation. Fig. 2*A* depicts a family of membrane currents associated with step depolarizations of 10–170 mV (20-mV steps) from the holding membrane potential of −70 mV. During a 30-min period after start of perfusion with 1×10^{-3} M SLA, the membrane current associated with a step depolarization of 70 and 170 mV underwent remarkable changes (Fig. 2*B*). The sodium and potassium currents were reduced progressively, and the potassium current exhibited a discernible inactivation even at the early stage of exposure to SLA, and the inactivation became more pronounced as time progressed. The potassium current initially rose almost normally, but soon decreased to a very low level. After 30 min of perfusion with SLA, the peak sodium and steady-state potassium currents were effectively decreased (Fig. 2*C*). The potassium inactivation proceeded in a manner greatly dependent upon the membrane potential, being accelerated and augmented with increasing depolarization. These effects were reversible upon washing with drug-free solution (Fig. 2*D*).

Local anesthetic without spin-labeled moiety (GS-01) did not induce a potassium inactivation, it simply suppressed both sodium and potassium currents (Fig. 3).

The time for the sodium current to attain its peak (T_p) was not significantly changed by 1×10^{-3} M SLA (Fig. 4*A*). On the contrary, T_p was greatly prolonged by 1×10^{-3} M GS-01 over the entire range of membrane potentials studied (Fig. 4*B*).

The current-voltage relationships for sodium and potassium are illustrated in Fig. 5. It is clearly seen that both sodium current (I_{Na}) and

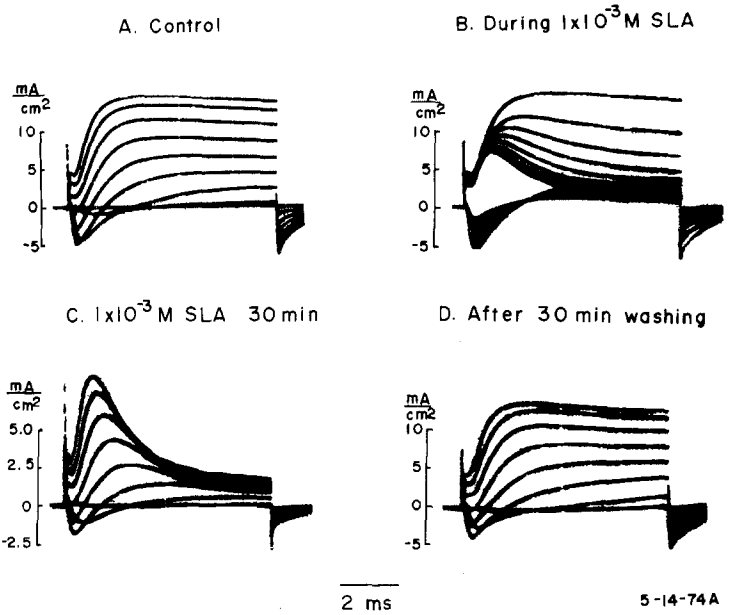

Fig. 2. Effects of external application of spin-labeled local anesthetic (SLA) on membrane currents associated with step depolarizations of 10 to 170 mV in 20-mV steps from the holding membrane potential of −70 mV (*A*, *C* and *D*). Record *B* represents superimposed membrane currents associated with step depolarizations of 170 mV (upper group of currents) and 70 mV (lower group) immediately before and during application of SLA. Note that the potassium current shows inactivation during application of SLA. Temperature, 13 °C

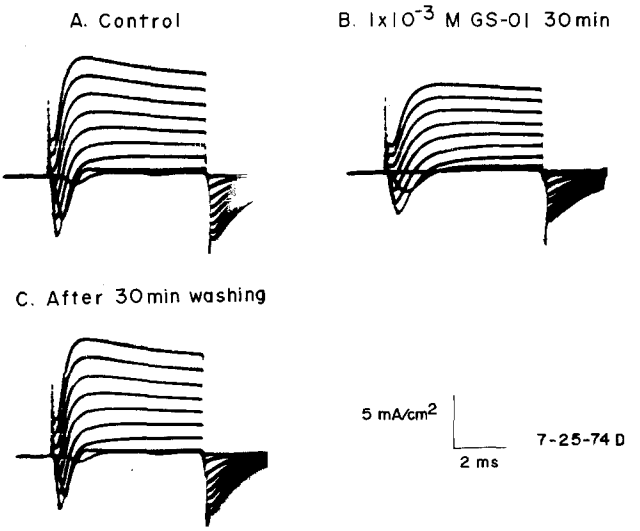

Fig. 3. Effects of external application of nonspin-labeled local anesthetic (GS-01) on membrane current associated with step depolarizations of 20 to 180 mV in 20-mV steps from the holding membrane potential of −70 mV (*A*, *B* and *C*). The slight drooping in the potassium current is due to the accumulation of potassium in the Frankenhaeuser-Hodgkin space. Temperature, 15 °C

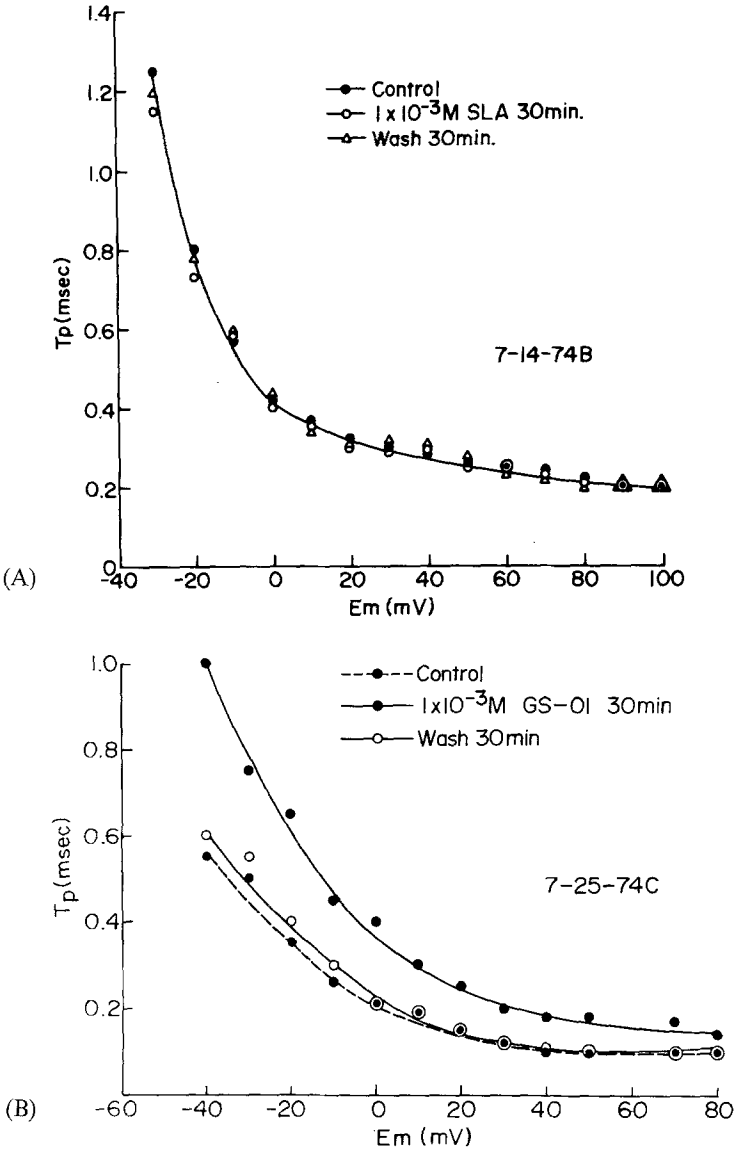

Fig. 4. (*A*) Time to peak transient current (T_p) as a function of the membrane potential before and during external application of 1 mM spin-labeled local anesthetic (SLA), and after washing with drug-free solution. Note that T_p is not significantly changed. (*B*) Time to peak transient current (T_p) as a function of the membrane potential before and during external application of 1 mM nonspin-labeled local anesthetic (GS-01), and after washing with drug-free solution. Note that T_p is prolonged by GS-01

potassium current (I_K) are decreased after application of SLA, and that the decrease in potassium current is drastic if measured 7 msec from the beginning of the depolarizing pulse when it attains a steady state.

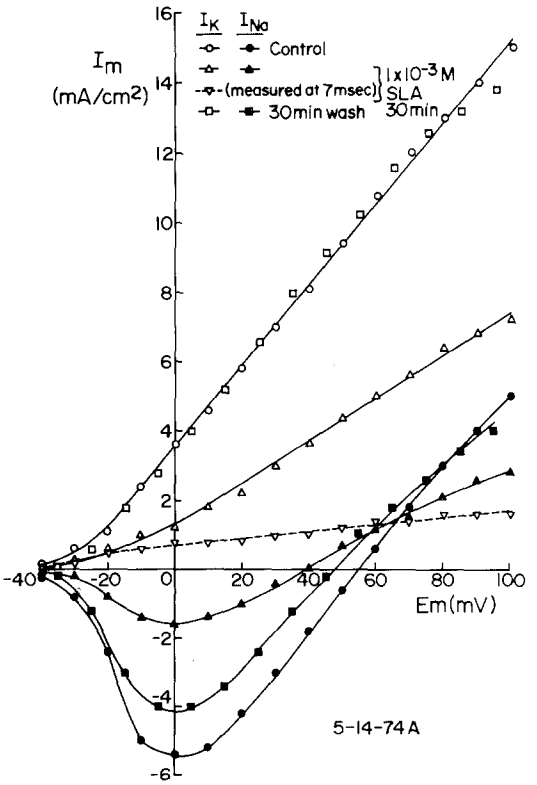

Fig. 5. Current-voltage relations for peak transient sodium current (I_{Na}) and potassium current (I_K) before and during application of 1 mM spin-labeled local anesthetic (SLA), and after washing with drug-free medium. The potassium currents during the application of 1 mM SLA were measured at the peak (△) and at 7 msec (▽)

The reversal potential for I_{Na} was shifted in the direction of hyperpolarization after application of SLA. No change in the reversal potential was observed after application of GS-01.

Effect on Membrane Conductances

The peak sodium conductance (G_{Na}) and the steady-state potassium conductance (G_K) were calculated as described in Materials and Methods. The mean values are given in Table 1.

The peak G_{Na} was suppressed to 48.6% of the control, the peak G_K to 48.5%, and the steady-state G_K measured at the end of a 7-msec pulse to 10% during application of 1×10^{-3} M SLA. GS-01, at the same concentration suppressed the peak G_{Na} and the steady-state G_K

Table 1. Application of 1×10^{-3} M spin-labeled local anesthetic (SLA) and nonspin-labeled parent compound (GS-01), and after (*A*) washing with drug-free medium. Maximum values of peak sodium conductance (G_{Na}), peak potassium conductance (G_K), and G_K at the end of a 7-msec pulse before (*B*) and during (*D*)

Conductances	Drug	*B* (mmho/cm^2)	*D* (mmho/cm^2)	*A* (mmho/cm^2)	*D*/*B* (%)	*A*/*B* (%)
Peak G_{Na}	SLA	102.5±7.60	49.8±4.61	87.6±10.9	48.6±2.96	85.3±6.32
	GS-01	99.3±9.43	78.1±7.57	97.8±10.2	78.9±4.20	99.9±1.12
Peak G_K	SLA	76.5±3.08	37.1±3.63	68.7±9.11	48.5±2.85	89.7±9.25
	GS-01	76.8±3.38	56.0±2.14	76.7±5.72	73.1±4.94	99.6±2.15
G_K at 7 msec	SLA	74.2±2.23	7.4±0.82	52.8±10.2	10.0±0.85	71.1±12.4
	GS-01	65.2±3.37	51.7±1.35	66.6±3.82	79.4±4.31	102.0±1.73

Data are expressed in terms of mean ±SD ($n=4$).

Table 2. Apparent dissociation constants (mM) of internally applied spin-labeled local anesthetic (SLA) and nonspin-labeled parent compound (GS-01) in suppressing peak sodium conductance (G_{Na}), peak potassium conductance (G_K), and G_K at the end of a 7-msec pulse

	SLA	GS-01	GS-01/SLA
Peak G_{Na}	0.35	1.6	4.73
	0.40	2.0	
	(0.38)	(1.8)	
Peak G_K	0.52	1.25	3.26
	0.48	2.00	
	(0.50)	(1.63)	
G_K at 7 msec	0.21	1.40	9.54
	0.23	2.80	
	(0.22)	(2.10)	

Data from two sets of experiments with the mean values in parentheses.

to 78.9% and 73.1% of the control values, respectively. The steady-state G_K measured at the end of a 7-msec pulse was suppressed to 79.4% of the control which was not significantly different from G_K measured at the peak value ($p > 0.20$).

Dose-Response Relation

The dose-response relations for SLA and GS-01 in suppressing the sodium and potassium conductance were studied by increasing the con-

centration of the test compounds cumulatively. The test compounds were perfused internally, partly because local anesthetics are known to block ionic conductances from inside of the membrane (Narahashi & Frazier, 1971) and partly because the small amounts of the compounds precluded external application which required a much larger volume of test solution than internal application. The apparent dissociation constants were estimated from the dose-response curves, and are given in Table 2. SLA was more potent than GS-01 in inhibiting peak G_{Na}, peak G_K and G_K measured at 7 msec, the potency ratios being estimated to be 4.73, 3.26 and 9.54, respectively.

Discussion

Spin-labeled local anesthetic inhibits both the peak sodium and the potassium conductance increases, and induces a marked potassium inactivation. The induction of potassium inactivation has been studied with a variety of pharmacological agents including tetraethylammonium derivatives (Armstrong, 1969, 1971), lobeline (Yeh & Narahashi, 1974*b*), quinidine (Yeh & Narahashi, 1974*c*), trihexyphenidyl (Wu, 1973), tropine derivatives and dibucaine (Blaustein, 1968; Narahashi, Moore & Poston, 1969), a veratrum alkaloid (Ohta, Narahashi & Keeler, 1973), sparteine (Ohta & Narahashi, 1973), and strychnine (Shapiro, Wang & Narahashi, 1974). The pharmacologically induced potassium inactivation is dependent on both membrane potential and time (Armstrong, 1969, 1971; Wu, 1973; Yeh & Narahashi, 1974*b*). It seems to require the opening of the potassium channel. Thus SLA will become a useful probe to study the mechanism of molecular interaction with the potassium channel.

Gargiulo, Giotta & Wang (1973) reported that several spin-labeled analogs of local anesthetics retained their local anesthetic action as evaluated by the duration and potency of surface anesthesia in the guinea pig cornea. Independently, Rosen (1974) observed that SLA possessed local anesthetic activity by measurements of the compound action potential of the frog sciatic nerve. Voltage clamp experiments have revealed important difference between SLA and its nonspin-labeled parent compound GS-01 in their ionic mechanism of action, which would have been undetected by other conventional techniques.

Three remarkable differences have been noted between SLA and GS-01: (1) SLA is more effective than GS-01 in suppressing the sodium and potassium conductance increases by either external or internal appli-

cation, indicating different intrinsic activities in blocking conductances. (2) SLA induces a potassium inactivation, whereas GS-01 is lacking this ability. The potassium currents clearly show inactivation when they are slightly suppressed in amplitude shortly after application of SLA (Fig. 2*B*). On the other hand, GS-01 does not induce any potassium inactivation with comparable degrees of amplitude suppression. (3) SLA has no effect on the time to peak sodium current, whereas GS-01 prolongs it despite its lower potency in suppressing the amplitude. GS-01 resembles procaine with respect to (2) and (3) above. It is possible that such differences occur between other spin-labeled local anesthetics and their non-spin-labeled parent compounds. This point should be borne in mind when spin-labeled local anesthetics are used as probes for the study of the molecular mechanism of action of local anesthetics.

In the past, electron spin resonance studies of biological membranes mainly focused on the interaction between the probe and bilayer membranes or resting axonal membranes. Giotta, Gargiulo & Wang (1973) have studied line shape changes of the electron resonance spectra of a series of spin-labeled local anesthetics interacting with resting axonal membranes in comparison with a known solvent mixture and they concluded that these compounds partition into the hydrophobic region of the membrane and that the extent of partitioning relates to the duration of local anesthetic action. In view of experimental observations indicating that the cationic form of tertiary amine local anesthetics interacts with sodium or potassium channels during excitation (Narahashi & Frazier, 1971, 1975; Strichartz, 1973), spin-labeled local anesthetics will become useful probes to study the gating mechanism of ionic channels.

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