

Stereoselective Inhibition of Neuronal Sodium Channels by Local Anesthetics

Evidence For Two Sites of Action?

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The objective of this study was to determine if the “tonic,” resting inhibition of Na^+ channels by local anesthetics results from binding at a site different from that for “phasic,” use-dependent inhibition. Stereoselective actions of four local anesthetics were examined in isolated frog peripheral nerve and single Na^+ channels. Using the sucrose-gap method on desheathed nerves, four actions of local anesthetics were assayed: 1) tonic depression of compound action potentials at low stimulation frequency (one per minute); 2) phasic depression of the compound action potential during trains of stimulation at 5, 10, and 20 Hz; 3) competitive antagonism of the reversible Na^+ channel activator veratridine assayed through the depolarization of the compound resting membrane potential; and 4) depression of the depolarization of the compound resting membrane potential initially induced by the irreversible channel activator batrachotoxin. For assays 1, 2, and 3, all local anesthetics showed a stereoselectivity, where *rectus*, or (+), enantiomers were more potent than *sinister*, or (–), enantiomers. In contrast, for the noncompetitive antagonism of veratridine's action and the depression of batrachotoxin-induced depolarization, also a noncompetitive interaction between anesthetic and activator, the (–) enantiomer was more potent than the corresponding (+) enantiomer. Blockade of single Na^+ channels activated by batrachotoxin in planar lipid bilayers was also stereoselective for the (–) enantiomer. These findings, along with previously reported voltage-clamp results, can be applied to infer properties of a local anesthetic binding site in activator-free channels. Local anesthetic molecules with more sharply angled shapes have stronger stereoselectivities than less angled, more planar drugs. The inversion of the stereopotency induced by the activators can be explained by either of two mechanisms. There may be two binding sites for local anesthetics, one of high and one of low affinity

and of opposite stereoselectivity; activators may change the conformation at the high affinity site, reducing its local anesthetic affinity below that of the usual low affinity site and thereby revealing the pharmacology of the weaker site. Alternatively, only a single binding site may exist and be conformationally altered by activators such that both anesthetic affinity and stereopotency are modified. In activator-free channels, however, a single, high-affinity binding site with a constant stereoselectivity can account for both tonic and phasic inhibition by local anesthetics. (Key words: Anesthetics, local: impulse blockade; Na^+ channel; stereopotency; veratridine.)

LOCAL ANESTHETICS block the propagation of nerve impulses by binding to receptors on the sodium (Na^+) channel and preventing normal function.¹ This binding appears to involve a single local anesthetic molecule,² and, based on the concentration of local anesthetic required to affect 50% inhibition of Na^+ current, the binding affinities to the site are not large, with apparent inhibition constants (K_i) $\geq 10^{-6}$ M.³ This implies a relatively poor fit and weak bonding between the drug and its binding site, a view supported by the broad variety of chemical structures that exhibit local anesthetic activity on Na^+ channels.

Binding affinities for local anesthetics depend on the conformation or state of the Na^+ channel.⁴ In a resting nerve, closed Na^+ channels may be in the resting conformation or the inactive conformation, with few, if any, in the open, conducting state. Local anesthetics bound to channels in the resting and inactive states at rest account for the “tonic” decrease of the action potential, measured when the nerve is stimulated at low frequency. Na^+ channels are also converted transiently from the resting to the open and inactive states through membrane depolarization. With more frequent repetitive stimulation, an increasing fraction of channels opens and inactivates compared to the same period at rest. As a result of the increased binding of local anesthetic to channels in the open and inactive states, each of which binds local anesthetic faster and to a greater extent than does the resting state,^{5–7} there is a further “phasic” (use-dependent) decrease in the drug-free Na^+ channel population and therefore a use-dependent inhibition of the action potential. The size of the phasic block relative to that of the resting, tonic block depends on the time course of appearance and disappearance of open and inactivated states and on their respective kinetics for local anesthetic binding.^{4,7,8}

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Local anesthetics also antagonize the actions of a class of drugs called channel "activators," which stabilize the Na^+ channel in the conducting, open conformation.⁹⁻¹² This antagonism by local anesthetic can be detected as an inhibition of activator-induced membrane depolarization,^{10,13} ionic Na^+ current,¹⁴ or ionic flux.^{11,15} In addition, the binding of one of these activators, batrachotoxin (BTX), to neuronal and cardiac Na^+ channels has been shown to be inhibited in a competitive yet apparently allosteric manner by local anesthetics.^{9,15,16} That is, rather than a spatially overlapping *steric* competition at a common site, the drugs appear to bind at separate yet interacting sites and thereby favor respective conformations of the channel that have much lower affinity for the other ligand.

The nature of the local anesthetic binding site is of both scientific and therapeutic interest. Because local anesthetics bind with different affinities to different channel states, the binding site must be coupled to the conformational changes that subserve channel "gating." Knowledge of this site's properties and location on the channel should provide useful information about the gating functions. The separate local anesthetic actions of impulse blockade, antiarrhythmia, and toxicity show a similar but not identical dependence on drug structure,^{17,18} and knowledge about the neuronal site may improve our ability to design drugs directed to selective therapies with reduced toxicity.

Stereoisomers of local anesthetics have been reported to show differences in potency for both the inhibition of normal channels and the antagonism of channel activators.^{10,19,20} The *rectus*, or (+), isomers are more potent than the *sinister*, or (−), isomers in inhibiting the normal Na^+ channel, as shown in this paper and previously by others.^{10,19-22} However, as we report here, when these Na^+ channels are saturated by membrane activators, the (−) isomer appears more potent than the (+) isomer.

We investigated this inversion of stereopotency by quantifying the ability of enantiomers of local anesthetics to block nerve action potentials in tonic and phasic modes and to antagonize the depolarization caused by the activator veratridine (VTD). In addition, we compared these stereopotency ratios to those for the blockade of the otherwise irreversible BTX-induced activation of channels in nerve and in lipid bilayer membranes.

Materials and Methods

ACTION POTENTIALS AND RESTING POTENTIALS: SUCROSE GAP

Under approval of the Harvard Medical Area Committee on Animals, sciatic nerves from the frog (*Rana pipiens*), which had been killed by decapitation after general anesthesia (2 ml of 1-mg/ml MS 222, Sigma Chemical

Co. St. Louis, MO), were excised, desheathed, and split longitudinally into two bundles. The preparation was mounted in a sucrose-gap chamber previously described.^{23,24} One end of the nerve was stimulated in drug-free Ringer's solution with Ag/AgCl or platinum electrodes, using square-wave supramaximal current pulses of 50- μs duration (WP Instruments Stimulator, model 1850A). Similar Ag/AgCl or platinum electrodes were used to record potential changes between two regions of the nerve separated by a sucrose gap. These potential changes were amplified (Metamatrix amplifier, model AK4700; MetaMetrics Co., Cambridge, MA) and recorded as either compound action potentials (CAP) on an a.c.-coupled, dual-beam storage oscilloscope (Tektronix 5113) or as the d.c. membrane potential (CRP) on a strip chart recorder (Cole-Palmer, model 1201-8001). The sucrose-gap technique²⁵ converts the extracellularly recorded bipolar compound action potential into a monophasic one (CAP) and permits the measurement of direct current changes proportional to the average resting potential of all axons in the nerve, the compound resting potential.¹⁰ All sucrose-gap experiments were done at 21–24° C.

Several limitations in data from sucrose-gap recordings limit any molecular interpretations. The relative depression of action potentials recorded by this method is not proportional to the occupancy of Na^+ channels,²⁴ so true dissociation constants cannot be determined. The nerve membrane is not controlled, as in voltage-clamp, and in fact is hyperpolarized from the resting value by sucrose; this not only changes the population distribution of Na^+ channels at rest (see Discussion, below) but also modifies the "tonic" inhibitory action of local anesthetics. During repetitive stimulation, CAPs of nerves in sucrose-gap, bathed by tetraethylammonium (TEA)-Ringer's solution as these are, show prolonged, albeit minor, depolarized afterpotentials that sum to give a sustained plateau.²⁶ The overall effect is one of a depolarized baseline coupled with large, transient spikes, thereby complicating any simple assignment of channel types to "tonic" versus "phasic" inhibition. Despite these limitations, the relative potencies of drugs can be determined with little ambiguity using sucrose-gap.

The nerve was first stimulated to determine a supra-maximal stimulus. Next, the flow of sucrose was begun, rapidly (20–30 $\text{ml} \cdot \text{min}^{-1}$) for 0.5 min to flush out the system, and then maintained at 2–3 $\text{ml} \cdot \text{min}^{-1}$ for the duration of the experiment. The test chamber held 320–350 μl solution. During the stabilization period, the solution in the test chamber was changed once with normal frog Ringer's solution and then twice with TEA-Ringer's solution, which greatly reduces outward potassium currents. This baseline period usually lasted 20–30 min. Afterward, stimulation at one per minute showed a steady

CAP and compound resting potential, each changing by less than 5% over a 30-min period. All drugs were tested in the sucrose-gap in TEA-Ringer's to avoid the complications from their actions on K^+ channels.²⁷

PLANAR BILAYER STUDIES OF SINGLE CHANNELS

Chemicals and Membrane Preparation

Synthetic phospholipids, phosphatidylcholine and phosphatidylethanolamine, were purchased from Avanti Polar Lipids (Birmingham, AL). Membrane vesicles from rabbit brain were prepared as previously described by Moczydlowski *et al.*²⁸ All membrane isolation methods were performed at 0–4° C. The vesicle preparation was suspended in 0.3 M sucrose, 10 mM HEPES, 0.2 mM EDTA, and 0.02% NaN_3 and stored at –70° C.

Planar Bilayers and Na^+ Channel Insertion

Planar bilayers (BLM) were cast from solutions of decane containing 13.4 mg/ml phosphatidylethanolamine and 6.7 mg/ml phosphatidylcholine, painted across 200- μ m-diameter holes in polyvinyl chloride partitions. Ionic currents were monitored at constant holding voltages using an EPC-7 Adams and List patch clamp (Medical Systems Corporation, Great Neck, NY). Standard aqueous solution was 200 mM NaCl, 0.2 mM EGTA, and 10 mM HEPES–NaOH, pH 7.4. Both sides of the bilayer contained identical aqueous solutions.

Insertion of Na^+ channels could be detected in the presence of 100 nM BTX added from the *cis* side of the bilayer, essentially as described by Krueger *et al.*²⁹ In general, plasma membrane vesicles (~10 μ g/ml final concentration) were added to the *cis* side of the bilayer and the voltages were alternated between –50 and +50 mV every 20 s to facilitate their incorporation. All voltages were defined as intracellular voltage, and the external face of Na^+ channels in the bilayer was defined as zero voltage. These Na^+ channels were blocked by tetrodotoxin in a voltage-dependent manner and were activated around –120 to –80 mV. Local anesthetics were applied internally, and the solution was stirred vigorously for about 1 min before recording began. All experiments were performed at $22 \pm 2^\circ$ C. Currents were filtered at 50–100 Hz, recorded at 100-Hz resolution, stored, and later analyzed by an AT computer using pCLAMP software (Axon Instruments Inc., Foster City, CA), which uses Marquardt-Levenberg algorithms for statistical estimates of open and closed time constants. Bilayer records containing more than one channel were discarded.

SOLUTIONS AND CHEMICALS

Compound resting and action potentials were measured in frog Ringer's solution containing (millimolar): NaCl

110; KCl 2.5; $CaCl_2$ 2.0; morpholino-propanesulfonic acid 5.0; the pH was adjusted to 7.20 with NaOH. The TEA-Ringer's solution contained, in addition, TEA chloride (12.0 mM) to block potassium currents.

VTD (Sigma) was dissolved in absolute ethanol to give a stock solution of 100 mM and then further diluted with TEA-Ringer's. The final maximum concentration of 1% (vol/vol) of ethyl alcohol, present when 1,000 μ M VTD was applied, by itself produced no change in the resting membrane potential or CAP. The stock solution of 100 mM VTD was made up fresh every 4 weeks and stored at –20° C. The stock solution was stable for this period. Dilutions were made from this stock solution for each day's experiment.

BTX, a gift from Dr. John Daly, National Institutes of Health, Laboratory of Biorganic Chemistry, was dissolved in dimethylsulfoxide to 1 mM and stored in a freezer at –20° C. This stock solution was further diluted with TEA-Ringer's to the concentration used in each experiment.

The stereoisomers of the local anesthetics, kindly supplied by Dr. Rune Sandberg of Astra Alab, Sodertalje, Sweden, are shown in figure 1. For the sucrose gap experiments, stock solutions were made up each day and diluted in TEA-Ringer's solution to the concentrations needed for each experiment.

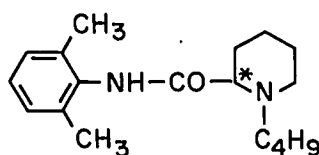
STATISTICAL ANALYSIS

The results are reported as means \pm SEM. To establish significant differences between parameters for two stereoisomers, a two-tailed Student's *t* test was applied, with $P < 0.05$ considered significant.

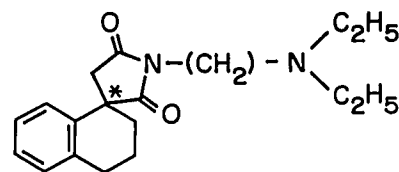
Results

COMPOUND ACTION POTENTIALS

All local anesthetics tested inhibited the CAP stereoselectively. The effects of bupivacaine stereoisomers on the CAP are shown in figure 2. The nerve was stimulated once per minute to allow observation of the changes of the CAP as the local anesthetic was added, exchanged, and removed. Bupivacaine, as the (+)-isomer, initially decreased the height of the CAP to a new steady state, usually reached within 20–30 min. In the experiment shown in figure 2, the solution of (+)-bupivacaine (200 μ M) was replaced by one containing an equal concentration of (–)-bupivacaine before steady-state was reached. The CAP was further diminished, but only transiently, and eventually recovered to an amplitude equal to ~60% of the pre-drug control. Subsequent replacement by 200 μ M (+)-bupivacaine led to a greater inhibition, which again was partially reversed (following another transient "overinhibition") when the (–)-bupivacaine solution was restored. Removal of all local anesthetic resulted in the recovery of the action potential toward its initial control value, a

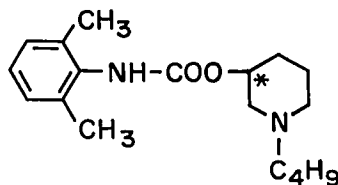


BUPIVACAINE

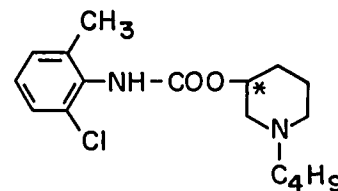


RAC 109

FIG. 1. Structures of local anesthetics used in this study. The asterisk indicates the asymmetric carbon in each molecule.



HS 38



HS 37

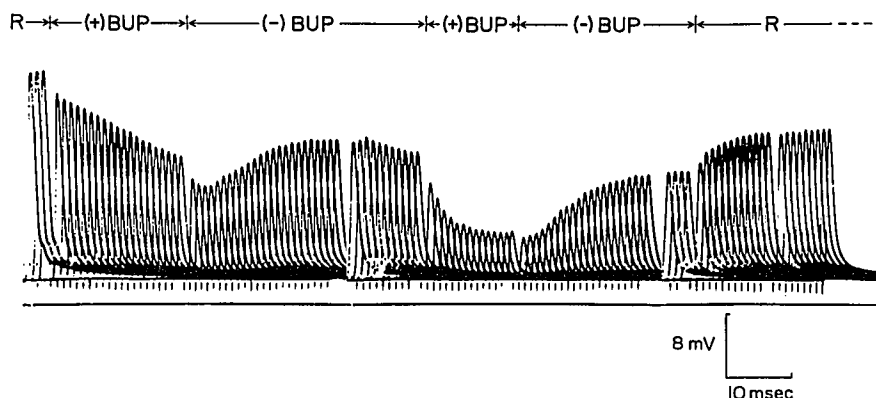
slow process that could take more than 1 h and that was often incomplete. The results shown in figure 2 demonstrate the greater tonic inhibitory potency of the (+)-isomer of bupivacaine.

In a different protocol, tonic inhibition was measured at increasing doses of one local anesthetic enantiomer on one nerve only. Then a second nerve from the same animal was used for the same test of the other enantiomer. Dose-response curves for the separately tested isomers were constructed from such experiments, as for HS38 in figure 3. In the range of 20–60% inhibition, the response has the same relative dose-dependence (slope) for both enantiomers, and the stereopotency ratio (+/–) is 4–5. From each separate experiment (in contrast to the summed, averaged data shown in fig. 3), we interpolated these dose *versus* tonic inhibition curves to determine an EC_{50} , the concentration for 50% inhibition, the average values of which are listed in table 1 (with the exception of HS37, for which EC_{30} values were used for the ster-

eopotency ratio since the highest density of data occurred in this inhibitory range). The tonic stereopotency ratios, listed in table 1 as the inverse ratio of the EC_{50} s, range from high values of 6–7 for HS37 to low ratios of 1–2 with RAC109 and bupivacaine.

When high-frequency bursts of stimulation were applied to the nerve in the presence of local anesthetic, an additional “phasic” decrement in the CAP was observed. At any one drug concentration, the steady-state phasic inhibition was greater at higher stimulation frequencies (fig. 4). Changes in the CAP during phasic stimulation at up to 20 Hz before drug application were less than 2%. Steady-state phasic inhibition, reached by the tenth pulse in a train of stimuli, also increased with drug concentration and showed a significant stereoselectivity. Figure 5A shows how the steady-state phasic decrement at 20 Hz, reported as the fraction of the control CAP before drug addition, varied as a function of the concentration of the (+) or (–) enantiomer of HS38. This dose-response curve was bi-

FIG. 2. A series of compound action potentials in frog sciatic nerve, stimulated at 1 min^{-1} , tracks the potency of bupivacaine (BUP) stereoisomers. Each sequential trace, sweeping at 10 ms of the time bar (lower right scale), is displaced incrementally to the right to provide a chronology of the effects as different solutions are perfused around the nerve. Initial inhibition by (+)bupivacaine ($200 \mu\text{M}$) is followed by partial recovery in less potent (–)bupivacaine ($200 \mu\text{M}$). The same order of potency occurs during the second sequential application of local anesthetics, despite the slow rundown of the control signal, as judged by incomplete recovery of control amplitude on washout with Ringer's (R).



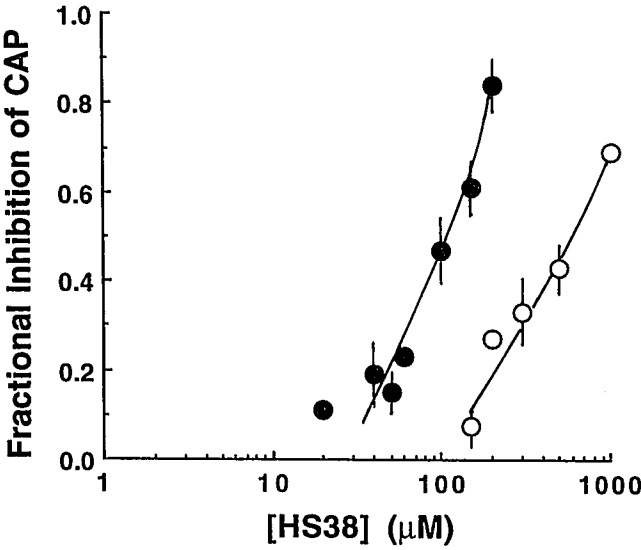


FIG. 3. Dose-response relationship for tonic depression of compound action potentials (CAP) by HS38. Curves are fits by eye. Solid circles = (+) isomer; EC₅₀ 100 μM; open circles = (-) isomer; EC₅₀ = 550 μM.

phasic; the sharp downturn of phasic inhibition at higher doses (approximately 100 μM (+)-HS38 and 500 μM (-)-HS38) resulted from the monotonically increasing tonic inhibition, which, by definition, limits the size of the phasic component to the remaining fractional CAP amplitude. At lower local anesthetic concentrations the phasic dose-response curves for (+)- and (-)-isomers were nearly parallel, indicating about a 10-fold higher phasic inhibitory potency of the (+) isomer over the (-), albeit with different corresponding degrees of tonic inhibition.

When phasic inhibition was compared in those situations where different concentrations of (+)-HS38 and (-)-HS38 were used to produce equal tonic inhibition, the (+) isomer was also more potent than the (-). The inhi-

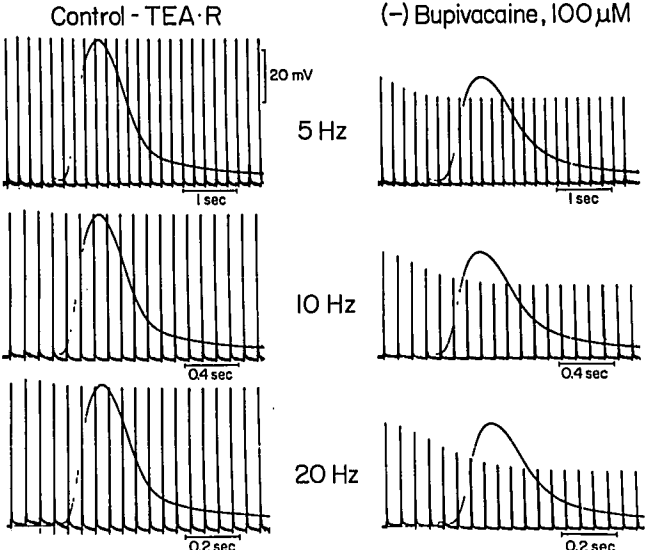


FIG. 4. Phasic inhibition of compound action potentials (CAP) by bupivacaine. In each panel, the response to a single stimulus is shown at the expanded time scale (1 ms for horizontal calibration bar) revealing both amplitude and shape of the CAP. Responses to repetitive stimulation are shown at slower time scales (noted by the values below each calibration bar). Left: CAP in control solution (TEA-Ringer's [TEAR]) stimulated at 5, 10, and 20 Hz. Right: (-)Bupivacaine (100 μM): the single CAP is depressed (tonic inhibition), and stimulation at higher frequencies produces a further, progressive decrease in the CAP (phasic inhibition).

bition also began to saturate as a function of stimulation frequency (fig. 5B). The difference in the phasic responses, superimposed on a background of equal tonic inhibition produced by a concentration of (-)-HS38 five times higher than that of (+)-HS38, can be used to calculate relative potencies for phasic inhibition. Using data like that from figure 5A, 100 μM (+)-HS38 yields 20% phasic inhibition at 5 Hz, in agreement with figure 5B; 500 μM (-)-HS38 yields 15% phasic inhibition at 5 Hz (fig. 5B),

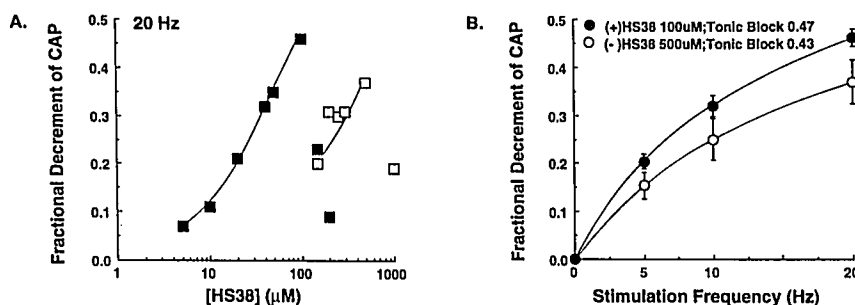
TABLE 1. Potency of Stereoisomers of Local Anesthetics: Inhibition of CAP and Competitive Antagonism of Veratridine

Local Anesthetic	Parameter	Measured Value		Potency Ratio (+/-)
		(+) (μM)	(-) (μM)	
Bupivacaine	EC ₅₀	135	220 *	1.6
	K _C	10.6 ± 1.0 (3)	32.4 ± 6.4 (3)†	3.0
RAC109	EC ₅₀	(I) 530	(II) 850	1.6
	K _C	61 (2)	278 (2)	4.5
HS38	EC ₅₀	112	525	4.7
	K _C	10.0 ± 1.2 (4)	39 (2)	4.1
HS37	EC ₅₀	30	200	6.6
	K _C	6.9 ± 1.1 (3)†	46.7 ± 10.7† (4)	6.0

Means ± SEM for n (in parentheses) independent measures.
* The fractional inhibition of the CAP at 200 μM (+)bupivacaine (0.81 ± 0.07%) differs significantly (P < 0.01) from the inhibition at

200 μM (-)bupivacaine (0.46 ± 0.05%).
† P < 0.01 comparing (+) and (-) stereoisomers using Student's t test.

FIG. 5. A: Dose-response relationships for phasic inhibition of compound action potentials (CAP) by HS38 stereoisomers at 20 Hz. Closed symbols = (+)HS38; open symbols = (−)HS38. As concentration of LA increases, phasic block increases to a maximum and then declines because the growing tonic block limits the possible extent of the phasic block. Curves were drawn by eye to the low concentration range of the data, collected from three separate experiments for each drug. B: The steady-state phasic block by HS38, measured at similar levels of tonic block, is plotted against the stimulation frequency. (−) Isomer is present at five times the concentration of the (+) isomer.



an effect that also would result from about 50 μM (+)-HS38. Such an equivalence demonstrates that under these more stringent conditions of equal tonic inhibition, the phasic stereopotency ratio (+/−) is still about 10.

For HS37, the phasic inhibition of the (+) isomer was similarly ten times more potent than that of the (−) isomer. An identical analysis of bupivacaine's phasic action shows a stereopotency ratio of 2.5–3, smaller than that of HS38 and HS37 but greater than bupivacaine's tonic stereopotency. In fact, all of the local anesthetics we tested have phasic stereopotencies for CAP inhibition greater than their tonic values.

When the local anesthetic was washed out with TEA-Ringer's, the response to tonic and phasic stimulation was assayed to demonstrate the reversibility of the inhibition. In all nerves, the tonic inhibition reversed by 80–95% on washing for 30–60 min, but a large residual fraction of phasic inhibition (approximately 25%) often remained. One explanation for this remaining inhibition is that residual local anesthetic binds more tightly to activated than to resting channels, such that the small degree of resting local anesthetic occupancy barely inhibits the CAP amplitude but further, phasically induced binding can manifest as a reduced impulse height.

ANTAGONISM OF MEMBRANE ACTIVATORS

Exposure of the sciatic nerve to VTD, a Na⁺ channel activator, resulted in a slow, spontaneous depolarization of the nerve membrane as recorded on a strip-chart recorder (fig. 6). Increasing concentrations of VTD produced increasing amounts of depolarization from which a dose-response relationship was determined (fig. 7; see ref. 10). Local anesthetics antagonized the depolarizing action of VTD. The response at VTD concentrations of 3–300 μM was smaller and generally slower in the presence of local anesthetic (fig. 6).

Consistent with the reversibility of VTD's action, the addition of local anesthetic after the depolarization had reached steady-state antagonized the signal as well as did incubation with local anesthetic before and during exposure to VTD (data not shown). Dose-response curves for the VTD-dependent depolarizations were shifted to the right in a parallel manner by local anesthetic (fig. 7), the EC₅₀ in the presence of local anesthetic exceeding that in its absence. From the size of such shifts, a dissociation constant for competitive inhibition (K_c) was calculated from equation 1¹⁰:

$$EC_{50}(LA)/EC_{50}(CTL) = 1 + [LA]/K_c \quad (1)$$

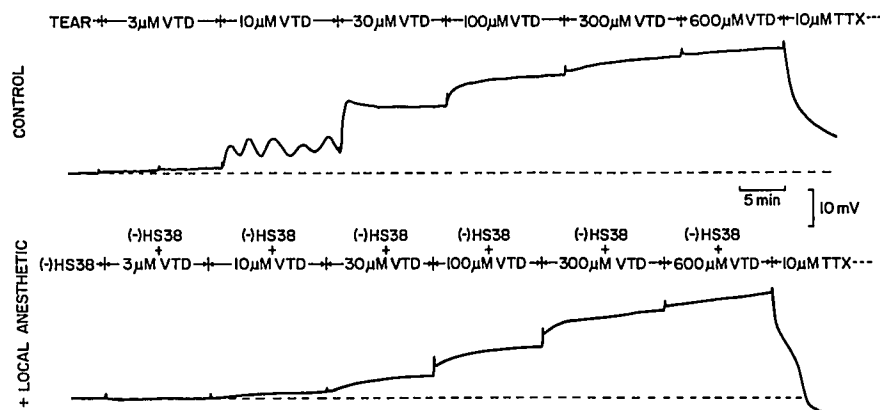


FIG. 6. The time course of changes in the compound resting membrane potential produced by increasing concentrations of veratridine (VTD) in the absence (top) and presence (bottom) of (−)HS38 120 μM. TEAR = TEA-Ringer's.

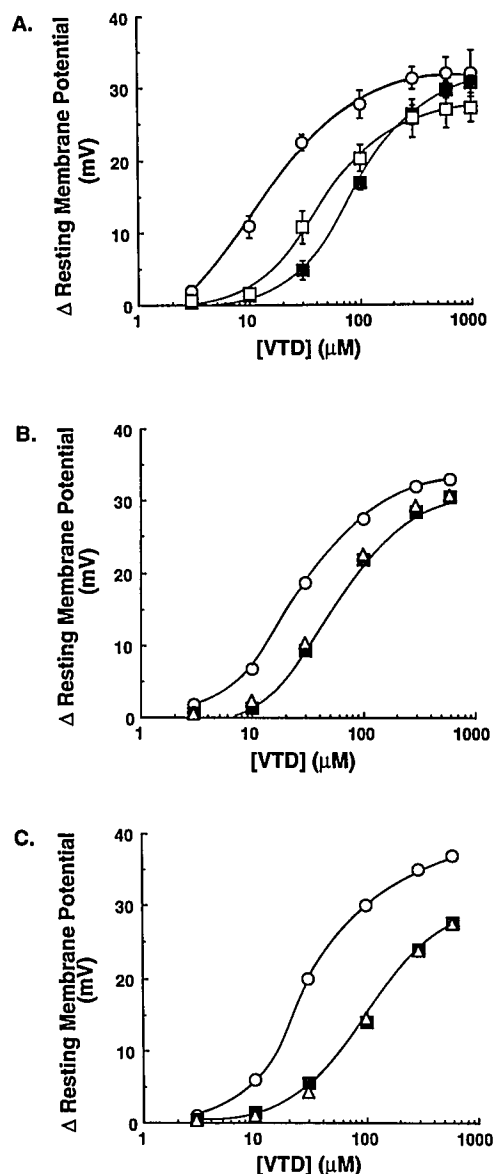


FIG. 7. Dose-response relationships for the veratridine (VTD)-induced depolarization of the compound resting potential. A: Open circles = control (Ringer's); solid squares = (+)-bupivacaine 50 μ M; open squares = (-)-bupivacaine 50 μ M. The curves were drawn by eye through data points showing the mean (\pm SE) from three separate experiments. B: Open circles = control; solid squares = (+)-HS37 5 μ M; open triangles = (-)-HS37 40 μ M. Data points are averages of three or four experiments. C: Open circles = control; solid squares = (+)-HS38 40 μ M; open triangles = (-)-HS38 120 μ M. Data points are averages of two to four experiments.

The competitive antagonism by local anesthetic was also markedly stereoselective; 5 μ M (+)-HS37 shifted the VTD dose-response curve to the same extent as did 40 μ M (-)-HS37 (fig. 7C). Table 1 lists the mean values of K_c for the four isomer pairs and their stereopotency ratios. For each local anesthetic, the (+) isomer was more potent than

the corresponding (-) isomer in competitively antagonizing VTD.

At high concentrations of VTD, from 600 μ M to 1,000 μ M, the dose-response curve was depressed in the presence of 50 μ M bupivacaine, apparently by a noncompetitive antagonism (fig. 7A). The extent of this effect with VTD was small, being 7.5% for (+)-bupivacaine and 18% for (-)-bupivacaine. Depression of the depolarizing response at saturating activator concentrations can be quantitated by a noncompetitive inhibitory dissociation constant (K_{nc}). The potency ratio for K_{nc} between (+) and (-) isomers, 0.43, being less than 1.0, was obtained through equation 2:

$$K_{nc} = [L](1 - y)/y \quad (2)$$

where y equals the fractional reduction of the saturated depolarization response and $[L]$ is the local anesthetic concentration. The (+) and (-) isomers of bupivacaine have K_{nc} values reversed in rank order from those found for tonic block, for phasic block of unmodified channels and for competitive antagonism of the VTD-induced depolarization.

The possibility that this noncompetitive antagonism could be observed only when a large proportion of the channels was activator-bound was tested by examining the stereoselective actions of local anesthetic on membranes modified by BTX, a virtually irreversible modifier of axonal Na^+ channels. When BTX (0.1 μ M) was added to the preparation, depolarization was extremely slow in onset (fig. 8). However, stimulation then induced a rapid depolarization of the membrane due to the increased fraction of open channels, which rapidly bind BTX.^{10,30} This depolarization was not reversed by washing with TEA-Ringer's. Addition of relatively high concentrations of bupivacaine (1 and 2 mM) antagonized the action of BTX. This depression of depolarization was partially reversed when the local anesthetic was removed from the bathing solution (fig. 8), an effect that would not occur if these inhibitory actions of local anesthetic were purely competitive, in which case the BTX would have been displaced from its binding site and washed away. The K_{nc} values for this depression of BTX-induced depolarization were 2.9 mM for (+)-bupivacaine and 0.83 mM for (-)-bupivacaine (table 2). The (-) isomer was more potent than the (+) isomer in this action, and the stereopotency ratio (+/-) identical to the one observed with VTD, was again reversed from that of activator-free channels.

BLOCKADE OF Na^+ CHANNELS IN PLANAR BILAYERS

To analyze further the actions of local anesthetics on BTX-modified channels in nerve, the inhibitory actions of local anesthetics on single Na^+ channels in planar lipid bilayers modified by BTX were studied.³¹⁻³³ Exemplary

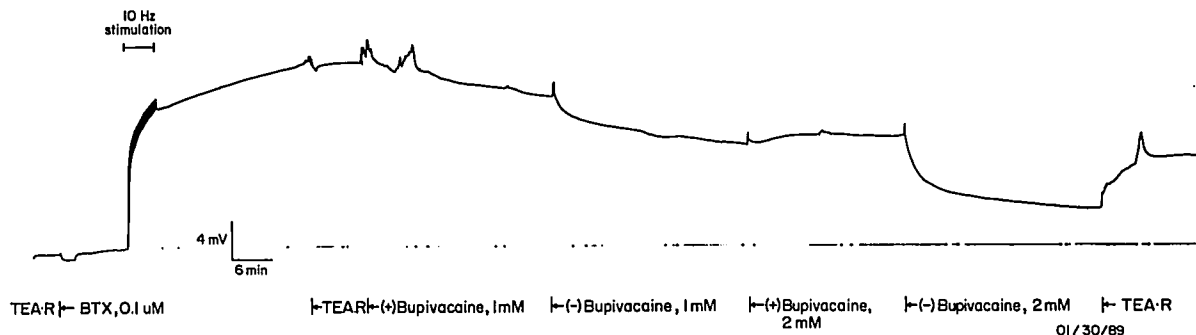


FIG. 8. Time course of the depolarization of the resting membrane potential by batrachotoxin (0.1 μ M) and its reversal by stereoisomers of bupivacaine at 1 and 2 mM. The brief period of stimulation 10 min after batrachotoxin addition accelerates the depolarization but does not alter its final level. TEAR = TEA-Ringer's; BTX = batrachotoxin.

traces are shown in figure 9. Control current traces show that BTX-activated Na^+ channels remain open most of the time ($\geq 97\%$) at voltages > -60 mV. The II isomer of RAC109 at 1 mM in the solution bathing the external surface yields an average blockage of about 40% (fig. 9A) with an estimated K_{nc} (equation 2) of 1.36 mM.³³ Direct kinetic analysis of these current traces is difficult to perform because of the poor time resolution of the bilayer system. For comparison, the stereoisomer RAC109-I is so much less potent that almost no block occurs at 1 mM; the estimated equilibrium dissociation constant exceeds 5 mM. Despite the lack of quantitative values, the potency ratio of less than 0.27 (I:II) is opposite that for tonic inhibition and competitive VTD antagonism (compare tables 1 and 2).

When bupivacaine's actions are similarly analyzed on BTX-modified single channels, a comparable stereopotency ratio is observed. (–)-Bupivacaine binds tightly enough to yield measurable occupancy times (fig. 9b); the mean fractional closed time with 300 μ M internal (–)-bupivacaine is near 90%, yielding a dissociation constant of 45 μ M. In contrast, the addition of 300- μ M-interval

(+)-bupivacaine produces infrequent and brief closed events yielding a corresponding dissociation constant of > 3 mM. These values are much greater than the corresponding K_{nc} values from the frog nerve—*e.g.*, 0.83 mM for (–) bupivacaine compared to 0.045 mM in the bilayer study. A substantial part of this 18-fold difference is attributable to the strong voltage-dependence of local anesthetic block of BTX-activated channels, a dependence that is independent of local anesthetic stereoselectivity.^{32,33} Block in the bilayer was measured at +50 mV, whereas the BTX-depolarized frog nerve, initially hyperpolarized below resting potential by the sucrose-gap (see Materials and Methods) may have reached -50 mV;¹⁰ this difference in potential corresponds to a factor of 10–12 in the apparent K_I . Nevertheless, despite the deviations from absolute K_D values in the frog nerve, the stereopotency ratio (+/–) in the BLM, 0.015, is still inverted from that observed in activator-free channels.

Discussion

The results of these experiments show that local anesthetics both inhibit action potentials and prevent or re-

TABLE 2. Noncompetitive Antagonism of Channel Activators by Stereoisomers of Bupivacaine and RAC109

LA	Concentration	Parameter	Measured Value		Potency Ratio (+/–)
			(+) (mM)	(–) (mM)	
Bupivacaine	50 μ M	K_{nc} -VTD*	0.81 \pm 0.30 (3)	0.35 \pm 0.18 (3)	0.43
	1 mM	K_{nc} BTX†	2.885 \pm 0.56 (4)	0.83 \pm 0.14 (4)	0.29
	300 μ M	K_D ‡	> 3	0.045	0.015
RAC109	1 mM	K_D ‡	I	II	< 0.28
			> 5	1.36	

Values are mean \pm SEM (n). Abbreviations are defined in text.

* Noncompetitive antagonism of VTD by bupivacaine. Dissociation constant calculated from depression of maximum of VTD dose-response curve in continuous presence of bupivacaine, using equation 2 ($0.05 < P < 0.10$).

† Noncompetitive antagonism of BTX by bupivacaine. Dissociation constant calculated from the reversal of BTX depolarization by bupivacaine using equation 2 ($P < 0.001$).

‡ Inhibition of BTX-modified Na^+ channel in lipid bilayer by bupivacaine. Dissociation constant derived from mean fractional closed time.

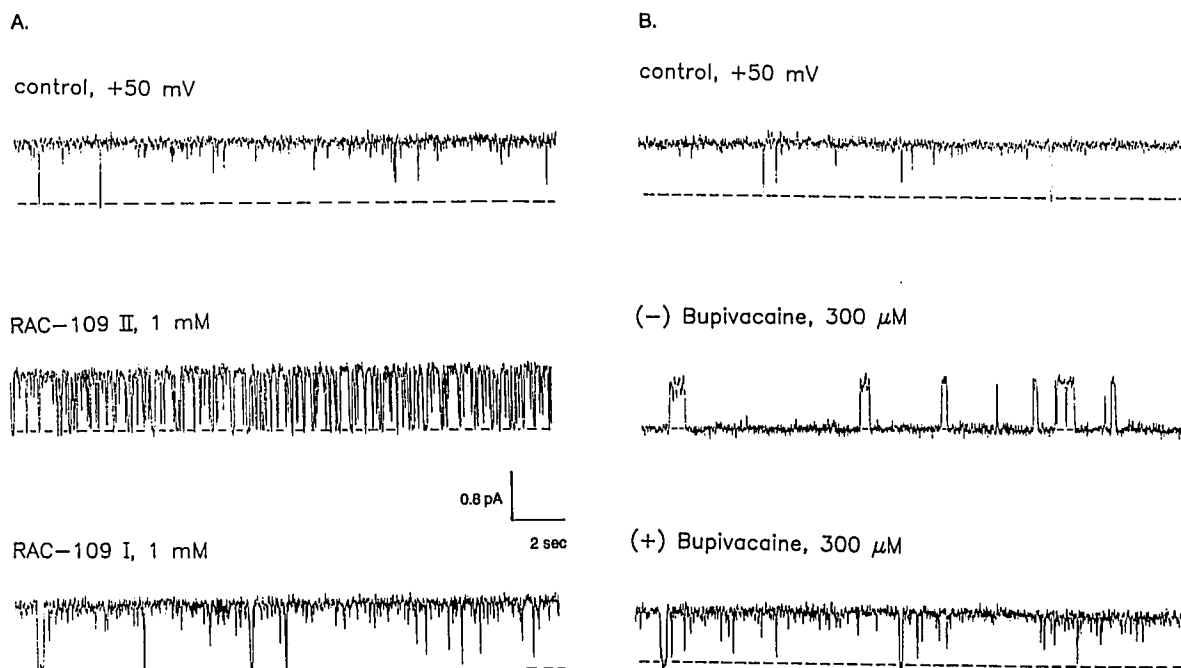


FIG. 9. Records of single batrachotoxin-activated Na^+ channels from rat brain in planar bilayers. Control current traces at +50 mV are shown on the top. Dashed lines indicate the 0 current line (closed channels). A: Frequent, brief channel closings occur in the presence of RAC-109II, whereas the incidence of such detectable events is much lower with its enantiomer, RAC-109I. The noisier baseline in RAC-109I compared to the control indicates that channel-blocking events too brief to be detected as full closings by this system are occurring with this isomer. B: Only (-) bupivacaine induces discrete closing events that can be easily measured. The mean closed time was estimated to be 1.8 s.

verse steady depolarizations induced by lower concentrations of VTD with the same stereopotency order: (+) > (-). However, when saturating concentrations of VTD are used or when the local anesthetics are applied to depress a depolarization induced by BTX or to block BTX-activated single Na^+ channels, then the stereopotency order is inverted: (-) > (+). The latter effects, which appear to be noncompetitive interactions between local anesthetics and Na^+ channel activators, also correspond to much weaker anesthetic binding of both enantiomers. This reduced local anesthetic affinity accompanied by an inversion of stereopotency seems to be a general action of membrane activators. In this discussion we will compare these findings with previously published ones, consider the kinetic basis for the stereopotency, and speculate on the structural features of the local anesthetics that produce stereopotency.

Stereoisomers of local anesthetics have been used previously to probe the topology of binding sites for local anesthetics on Na^+ channels. These optical isomers have identical bulk physicochemical properties and have been shown to have the same uptake into nerve and other biologic membranes.³⁴ Thus, differences in potency for these isomers reflect differences in binding to the receptor.

In the experiments reported here, the (+) enantiomer of all four local anesthetics tested was more potent than

the corresponding (-) enantiomer. Similarly, Akerman* found the same stereopotency order for these four compounds in the reduction of biphasically recorded CAPs, and a similar stereopotency ratio to the one seen here for RAC109 was reported by Rando *et al.* using an identical sucrose-gap.¹⁰ In voltage-clamp studies of frog myelinated nerve (at 11° C), Hille *et al.*²⁰ observed that 125 μM RAC109-I blocked Na^+ currents more than did 250 μM RAC109-II, corresponding to a tonic stereopotency ratio > 2. On the other hand, Yeh²¹ found that in internally perfused squid giant axons at rest, RAC109-I and -II were equipotent in producing tonic block. One possible explanation for the disagreement between the results in squid and frog lies in the differences in degree of inactivation of the Na^+ channels at rest between the two preparations. The resting balance between the resting and inactive states is dependent on the membrane potential; hyperpolarization will increase the proportion of channels in the resting state, and depolarization will increase that in the inactive state. As mentioned before, local anesthetics have a lower affinity for the resting than for the inactive state, demonstrating a conformational change at the drug binding

* Akerman B: Studies on the relative pharmacological effects of enantiomers of local anesthetics with special regard to block of nervous excitation. Astra Uppsala (Research Reports), 1973.

site between these two states. In the experiments on frog nerve,^{10,20,35} resting inactivation was always present under test conditions, but in the study on squid,²¹ the membrane was held at potentials negative enough to remove all channels from the inactive state at rest. One possible explanation for the difference in tonic stereoselectivity is that the inactive state binds local anesthetic stereospecifically, whereas the resting state does not. Differences between species should also be considered.

The phasic inhibition that develops under rapid stimulation shows an enhanced stereoselectivity over that of the tonic level. The (+) isomer of all four local anesthetics was always more potent phasically than the corresponding (−) isomer, and the phasic stereopotency always exceeded the tonic. For example, both HS37 and HS38 have phasic stereopotency ratios of about 10 (fig. 5), whereas the tonic ratios are 6.6 and 4.7, respectively (table 1).

A previous analysis of phasic inhibition of neuronal Na⁺ current²¹ showed that binding of bupivacaine to “activated” channels (including preopen and open conformations) was faster for the (+) isomer, whereas dissociation from these states showed little stereoselectivity. Dissociation from closed channels was significantly slower (about 2.5 times) for (+)- than for (−)-bupivacaine. Similar differences in dissociation rate have been reported for bupivacaine in cardiac muscle.³⁶ In the case of the carbamyl-linked local anesthetic, HS37, acting on the neuronal Na⁺ current, like bupivacaine, the (+) isomer bound faster to and dissociated slower from activated states than did the (−) isomer. However, unlike bupivacaine, (+)-HS37 dissociated much faster (approximately four times) than (−)-HS37 from closed channels in the neurons.³⁵ This would selectively disfavor phasic inhibition by (+)-HS37, so that the larger stereoselectivity of steady-state phasic inhibition (~10) means that (+)-HS37 is binding to activated channels more than ten times faster than (−)-HS37.

Yeh²¹ found that the stereoselectivity of phasic inhibition in the squid was voltage-dependent. As with tonic inhibition, below −60 mV there was no stereoselectivity for RAC109. In the range from −60 to +40 mV, however, stereoselectivity developed and, furthermore, increased as the test potential became more positive. Both of these observations are consistent with local anesthetics binding stereoselectively to “activated” (open and preopen) states of the Na⁺ channel to produce phasic inhibition by brief depolarizations. This is because significant channel activation in squid (*Loligo pealeii*) does not occur at potentials negative to ~60 mV,³⁷ although inactivation develops extensively from −80 to −60 mV.³⁸

The stereopotent actions of RAC109 on Na⁺ channels in cardiac muscle are similarly frequency- and voltage-dependent to that in nerve. Use-dependent block can result from drug binding to several different channel states. Clarkson²² found two components to use-dependent block: a fast component, with a half-time to reach steady-

state of 2–3 ms, and a slow component, with a half-time of 14–15 s. He suggested that this fast “activation” block may be primarily due to binding of local anesthetic to open Na⁺ channels. Applying a version of Starmer’s “guarded receptor hypothesis,” Clarkson concluded that resting inhibition of cardiac Na⁺ current by RAC109 was not significantly stereoselective, but that both the rapid and slow phases of use-dependent block showed stereoselectivity. RAC109-I bound faster than RAC109-II to activated channels (rapid phase) and dissociated slower; binding to inactivated channels was equally rapid for both enantiomers but II dissociated more rapidly than I. Dissociation of RAC109 from resting channels was also significantly faster for isomer II, analogous to earlier observations on bupivacaine.^{35,36} Interestingly, contrary to the results in squid, Clarkson found no difference in the steepness of voltage-dependence block in heart by RAC109-I versus RAC109-II.²²

Thus, in all previous studies of activator-free membranes the (+) enantiomers of local anesthetics have been more potent for phasic inhibition than the (−) enantiomers. In some cases, resting inhibition may have shown no stereopotency, but this was not so in amphibian nerve, as found in the present study. When the kinetics of phasic inhibition by bupivacaine and RAC109 are compared, the stereopotencies are strikingly similar. Even though these drugs have their asymmetric carbons at opposite ends of the molecule (fig. 1), both have (+) isomers that bind more rapidly and dissociate more slowly than their corresponding (−) isomers. In contrast, although the (+) isomer of HS37 binds faster than the (−) enantiomer, it also dissociates much faster from closed channels.

Structural comparisons of bupivacaine, RAC109, and HS37 and HS38 together show that the first two have a shallower angle between the two ring systems in the molecule than do the last two. In bupivacaine, where an amide bond links the piperidine ring at an equatorial conformation, this results in a smaller angular difference between the substituted piperidine rings of the (+) and (−) isomers when the aromatic xylylide moieties are aligned identically. A similar geometry occurs in RAC109, where, because of the flexibility of the 3° amine moiety, the eccentricity between (+) and (−) forms is not as large as it would be if the alkyl amine substituent were rigid as in bupivacaine or HS37. In contrast, the piperidine ring of HS37 and HS38 is linked to the xylylide group through a carbamyl bond that favors the axial piperidine ring attachment, thus placing the N-butyl substituent at a steeper angle from the plane of the xylylide moiety. The resulting angular difference between the tertiary amine moieties of (+) and (−) enantiomers of HS37 (and HS38) is much greater than that between the corresponding bupivacaine isomers.

Fits of the local anesthetics to the binding site in activator-free channels appear to be sensitive to these differ-

ences. Binding constants, determined by competitive antagonism of VTD, for the (+) enantiomers of bupivacaine, HS38, and HS37 decrease in that order, at 11 μM , 10 μM and 7 μM , respectively (table 1), whereas those of the (–) enantiomers show the opposite trend: 32 μM , 39 μM , and 47 μM , respectively. The net stereopotency ratio (+/–) in this series increases from 3 to 4–6 because the (+) isomers bind more tightly as the (–) isomers bind more loosely. The more eccentric molecules show greater stereoselectivity.

Dissociation rates from closed channels after phasic block are also markedly different for bupivacaine and HS37.³⁵ The (+) enantiomers dissociate at similar rates, 0.62 s^{-1} and 0.92 s^{-1} for bupivacaine and HS37, respectively, but the (–) enantiomers differ strongly and oppositely in this dissociation, having respective rates of 0.14 s^{-1} and 0.022 s^{-1} . That the (–) enantiomer of bupivacaine dissociates six to seven times faster than the (–) form of HS37 speaks for the substantially greater binding affinity of that particular local anesthetic's (–) conformation. In a previous study of frog myelinated nerve,³⁹ we showed a steep dependence on molecular weight for dissociation of charged local anesthetics from the closed Na^+ channel, indicative of a narrow crevice for drug escape.⁴⁰ The present results and those of Guo *et al.*³⁵ with HS37 and bupivacaine show that more sharply angled local anesthetics in the (+) conformation escape from this crevice a little faster than the less angled ones, but that the more sharply angled ones in the (–) conformation have a much more difficult time leaving. However, only part of the difference in departure rates may be steric; the remainder may be due to the difference in binding energy between local anesthetic and channel site.⁴¹

In the presence of channel activators, the stereopotency ratios are inverted, and, in addition, both enantiomers of the local anesthetics bind more weakly. The affinity of (+)-bupivacaine is reduced about 300-fold by the presence of BTX, whereas that of (–)-bupivacaine falls about 30-fold (compare tables 1 and 2). We believe that the local anesthetic depression of activator-induced depolarizations in nerve usually results from the sum of competitive and noncompetitive antagonisms. The ratio of the second to the first action is least in the case of VTD's depolarization of nerve, where relatively easy activator dissociation invites competitive inhibition. This assay shows the weakest stereopotency (table 2). By comparison, blockade by local anesthetics of Na^+ channels activated by BTX in the planar bilayer relies almost exclusively on noncompetitive inhibition, appearing as first-order binding to open channels.^{31,32,42} The bilayer assay also shows the strongest stereoselectivity.

Previous reports noted a stereopotency (I/II) for RAC109's inhibition of BTX-activated flux of >1 .^{9,15} This order agrees with that for the competitive actions listed in table 1 and probably reflects the displacement of re-

versibly bound BTX in these systems, a displacement that is the predominant mode of inhibition of the flux measurements that take long to assay. The fast, noncompetitive actions of local anesthetics cannot be detected easily in the flux assays.

Two different molecular mechanisms can explain the inversion of local anesthetic stereopotency. In the persistent presence of channel activators, the local anesthetics express their relatively weak, noncompetitive inhibitory action. Appearing as an open channel block, these effects are most easily interpreted as occlusion of the channel's open pore.^{32,33} The tighter binding of local anesthetics that accounts for the competitive antagonism of activator actions and for the tonic and phasic inhibition of activator-free channels has a totally different stereopotency and may result either from local anesthetic binding to a different site, away from the channel's pore, or from a conformational change occurring at the nominal local anesthetic binding site due to allosteric actions of BTX. When activators bind to Na^+ channels, most of the channel functions are altered, including activation and inactivation, ion selectivity, and the binding of other drugs and neurotoxins.⁴³ According to the first explanation, in activator-free channels the normal high affinity binding of local anesthetics would prevent channels from opening at relatively low local anesthetic concentrations, obscuring the channel occlusion that would block the pore but only at the low affinity site at much higher concentrations. According to the second explanation, there is only one binding site for local anesthetic, and it is modified by activators such that it binds local anesthetic with weaker affinity and altered stereoselectivity. At present we cannot discriminate between these two alternatives. We conclude, however, that characterizations of the local anesthetic binding to Na^+ channels made on the basis of observations of BTX-modified channels can only be extended to the pharmacology of normal Na^+ channels with great caution.

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