Kinetics of local anesthetic inhibition of neuronal sodium currents
pH and hydrophobicity dependence

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ABSTRACT This study assesses the importance of local anesthetic charge and hydrophobicity in determining the rates of binding to and dissociation from neuronal Na channels. Five amide-linked local anesthetics, paired either by similar pK_a or hydrophobicity, were chosen for study: lidocaine, two tertiary amine lidocaine homologs, a neutral lidocaine homolog, and bupivacaine. Voltage-clamped nodes of Ranvier from the sciatic nerve of *Bufo marinus* were exposed to anesthetic externally, and use-dependent ("phasic") block of Na current was observed. Kinetic analysis of binding (blocking) rates was performed using a three parameter, piecewise-exponential binding model. Changes in extracellular pH (pH_o) were used to assess the role of drug protonation in determining the rate of onset of, and recovery from, phasic block. For those drugs with pK_a's in the range of pH_o tested (6.2–10.4), the forward binding rate during a depolarizing pulse increased at higher pH, consistent with an increase in either intracellular or intramembrane concentration of drug. The rate for unbinding during depolarization was independent of pH_o. The dissociation rate between pulses also increased at higher pH_o. The pH_o dependence of the dissociation rate was not consistent with a model in which the cation is trapped relentlessly within a closed channel. Quantitative estimates of dissociation rates show that the cationic form of lidocaine dissociates at a rate of 0.1 s^{-1} (at 13°C); for neutral lidocaine, the dissociation rate is 7.0 s^{-1}. Furthermore, the apparent pK_a of bound local anesthetic was found to be close to the pK_a in aqueous solution, but different than the pK_a for "free" local anesthetic accessible to the depolarized channel.

INTRODUCTION

This study examines the effect of pH and hydrophobicity on the rates of interaction of local anesthetics (LA) with Na channels. Because most clinical local anesthetics are tertiary amines with pK_a's in the range of 7 to 10, there are significant fractions of both the uncharged free amine form and the cationic protonated form available at physiologic pH. When a LA is applied outside a cell, the extracellular pH, by affecting the degree of protonation of drug, can strongly affect the potency for block of Na currents (Strobel and Bianchi, 1970; Narahashi et al., 1970). It is now well established that the binding site(s) for LA in nerve and skeletal muscle is not directly accessible from the outside of the cell membrane (Frazier et al., 1970; Strichartz, 1973; Hille, 1977*a*). An externally applied anesthetic must therefore be able to penetrate the cell membrane to exert an effect. This has been demonstrated most vividly by experiments with the permanently cationic, lipid insoluble quaternary lidocaine analogs QX-314 and QX-222. These compounds block Na channels effectively at concentrations of 0.1–1 mM when applied inside squid axons (Narahashi et al., 1970; Frazier et al., 1970; Cahalan, 1978; Yeh and Tanguy, 1985), myelinated nerve fibers (Strichartz, 1973; Khodorov et al., 1976) and skeletal muscle (Schwarz et al., 1977), but have very low potency when applied outside the axon.

Two modes of Na current (I_{Na}) inhibition by local anesthetics have been operationally defined: tonic block, measured as a decrease in I_{Na} in the absence of "recent" activiation, and phasic or use-dependent block, measured as a decrease in I_{Na} during repetitive activation. The mechanism(s) for tonic block remain controversial; in contrast, there is good evidence that the phasic blocking actions of LAs are caused by binding at a site on or near the sodium channel (Strichartz, 1973; Courtney, 1975; Hille 1977*a*, *b*; Schwarz et al., 1977; Cahalan and Almers, 1979). Studies of the pH-dependent rates of onset of, and recovery from, phasic block with 3° amine LAs (Hille, 1977*a*, *b*; Schwarz et al., 1977; Courtney et al., 1978) show that both onset of and recovery from block is more rapid at alkaline pH_o; block by neutral benzocaine and quaternary QX-572 are little affected by changes in pH_o, suggesting that pH affects the drug charge and not the receptor. Schwarz et al. proposed the following model: (*a*) Charged forms of LA access a binding site within the channel via a hydrophilic route, and require an open channel to bind and unbind. (*b*) Neutral forms bind to the

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same site, but can access the site by lateral diffusion through the bilayer, and can bind to and unbind from any state of the channel. (*c*) External protons can pass through the external mouth of the channel to alter the state of ionization of amine drugs on the receptor. A similar physical model for drug and proton access has been described in the context of a guarded receptor hypothesis (Starmer et al., 1984; Starmer and Courtney, 1986), incorporating most of the features of Schwarz et al. but without a modulation of the receptor affinity for drug.

Much work in recent years has focused on the rate of recovery from phasic block with various anesthetics (Courtney et al., 1978; Courtney, 1980*a*, *b*; Grant et al., 1980, 1982; Broughton et al., 1984; Clarkson and Hondeghem, 1985; Moorman et al., 1986), in part because the recovery rate is easily measured, and in part because it is thought to play an important role in the antiarrhythmic actions of many LAs. From these studies, it is apparent that the extent of drug ionization, as estimated from the pK_a in free solution and pH_o, cannot alone account for the wide range of recovery rates observed among anesthetics of similar free solution pK_a. One possibility is that the pK_a of the bound drug is shifted by the binding interaction, the extent and direction of the shift depending on the particular LA (Moorman et al., 1986). In addition, Courtney (1987) has proposed that both drug solubility in the cell membrane and the molecular dimensions of the drug molecule play as important a role as drug charge in determining the overall rate of recovery. That is, drugs of low lipid solubility will have difficulty in exiting the binding site via the membrane pathway; similarly, drugs of large molecular dimensions will experience a diffusion barrier that retards their escape from the closed channel.

The present study was designed primarily to assess the relative importance of these physicochemical parameters in determining the rate of recovery from phasic block in the voltage-clamped node of Ranvier. A series of lidocaine homologs, paired either by pK_a or by lipid solubility (estimated from octanol/aqueous partition coefficients), was tested. These drugs incude a novel, neutral lidocaine homolog, 5-hydroxyhexano-2',6'-xylidide (5-HHX), for which tonic and phasic Na current inhibition has been described (Chernoff and Strichartz, 1989*a*).

Extracellular pH was used to modulate the degree of drug protonation. Based on the work of Courtney (1975), a three-parameter model was developed (see preceding paper [Chernoff, 1990]) to estimate the rates of binding to, and dissociation from, the LA receptor during phasic block. Using this model, we were able to estimate quantitatively the fractional binding and unbinding of drug occurring during a depolarizing pulse. The effective "on-rate" (fractional binding per pulse) was strongly modulated by pH_o, consistent with titration of drug charge; the effective "off-rate" was only weakly dependent on pH_o.

To probe the pH dependence of the rate of recovery from phasic block in more detail, a separate three-state, four-parameter model is introduced. Estimates of these model parameters for lidocaine and bupivacaine yield two significant findings: (*a*) the apparent pK_a of bound drug is close to the free solution pK_a, and (*b*) the protonated form of anesthetic is able to depart, albeit more slowly than the neutral form, from the closed channel. The measured recovery rates for all five drugs tested are in general agreement with Courtney's (1987) structure-activity correlations: pK_a appears to be an important, but not the sole determinant of the rate at which LAs depart from closed Na channels. An abbreviated form of these results has been communicated previously (Chernoff and Strichartz, 1988, 1989*b*).

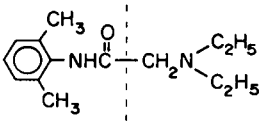
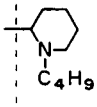
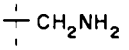
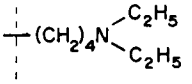
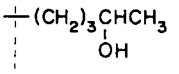
MATERIALS AND METHODS

Single myelinated nerve fibers from the sciatic nerve of toad (*Bufo marinus*) were isolated and voltage-clamped at 13°C as described in the preceding paper. The internodes were cut in a solution composed of (in millimolars): 120 CsCl, 5 NaCl, 10 3-(*N*-morpholino)-propanesulfonic acid (MOPS), titrated to pH 7.3 with tetramethylammonium (TMA) hydroxide. This solution blocked >95% of the "delayed rectifier" K^+ currents. The external solution contained (in millimolars): 115 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 10 MOPS, titrated to pH 7.3 with TMA · OH. In some experiments, 12 mM tetraethylammonium (TEA) chloride was added to the external solution to further inhibit K^+ currents, with no apparent effects on anesthetic block of sodium channels. In experiments at other levels of external pH, 10 mM of 2-[*N*-morpholino]ethanesulfonic acid (MES: pH 6.2), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS: pH 8.2), 2-(cyclohexylamino)ethanesulfonic acid (CHES: pH 9.4), or 3-[cyclohexylamino]propanesulfonic acid (CAPS: pH 10.4) was substituted for MOPS. Internal pH was assumed to be constant during external pH changes, based on the buffer capacity of the internodal solution and the low membane permeance of the zwitterionic buffers. However, due to the technical difficulties involved, internal pH was not measured.

Drug stocks and chemicals

Lidocaine-HCl and *d*-bupivacaine-HCl (the (+) optical isomer of bupivacaine) were the gift of Dr. Bertil Takman, Astra Pharmaceuticals, Westboro, MA. Glycylxylidide-HCl (GX), 5-(diethylamino)pentano-2',6'-xylidide · HCl (PRG-030), and 5-hydroxyhexano-2',6'-xylidide (5-HHX) were generously provided by Dr. Paul McMaster, College of the Holy Cross, and Dr. Eugene Byrnes, Assumption College, both in Worcester, MA. The syntheses of the last three compounds are described in Tenthorey et al. (1981). Drug structures, molecular weights, octanol/aqueous partition coefficients, and concentrations producing 50% inhibition of peak I_{Na} when applied extracellularly at a holding potential of −80 mV are shown in Table 1. Concentrated stock solutions were either prepared fresh the day of an experiment or stored at −15°C in small aliquots. Individuals aliquots were thawed as needed and stored at 4°C for no more than 7 d before use. Drugs were diluted into the external solution as needed; dilutions were 1:100 or greater.

TABLE 1 Physicochemical properties of lidocaine homologs

Drug	Mol wt	Structure	pK_a	P^o	P^+	IC50*
						μM
Lidocaine	234		8.19‡/8.41§	304¶	0.06¶	200
Bupivacaine	288		8.21‡/8.34§	2565¶	1.5¶	25
Glycylxylidide	178		8.02‡	3.8¶	ND	800
PRG-030	276		10.56‡	263¶	ND	1000
5-HHX	235		<2‡	9¶	ND	2000

*Concentration producing a 50% tonic decrease in P_{Na} at $E_{hold} = -80$ mV, $T = 13°C$. ‡At 25°C in 0.17 M NaCl buffer. §At 13°C in 0.17 M NaCl buffer. ¶Relative concentrations between octanol and buffer, 25°C. ¶Relative concentrations between octanol and H_2O, 37°C. ND, not determined.

Protocols

Details of the myelinated nerve voltage clamp, chamber dimensions, and data acquisition methods are given in the previous paper (Chernoff, 1990). Drugs were applied extracellularly, in concentrations previously determined to produce about 50% tonic block of peak I_{Na} at pH_o 7.3. A constant concentration of drug in the external solution was maintained, while pH_o was varied under conditions of constant ionic strength and buffer strength by substitution of one zwitterionic buffer for another in the external solution.

After solution changes, sufficient time was allowed (usually 5–10 min) for tonic block to reach equilibrium before phasic block testing was begun. The phasic block protocol consisted of repetitive pulses from the holding potential (−80 mV unless noted otherwise) to 0 mV, each pulse lasting 16 ms. These conditions produced inward Na currents, near-maximal activation of the sodium permeability, and nearly complete inactivation by the end of the pusle. After applying a pulse train, the fiber was rested for a period greater than three times the time constant for recovery from phasic block, this interval depending on the particular drug and the pH_o. A new pulse train was then applied at a different stimulation rate. In most cases, three or more pulse trains were used, at stimulation rates ranging over at least one order of magnitude. The peak inward sodium current for each pulse in a train was measured, normalized by the size of the first peak in the train, and plotted vs. pulse number. A three-parameter, piecewise-exponential "Courtney" model (see preceding paper) was fit to the data from all the pulse trains simultaneously, using a nonlinear least-squares algorithm (Colquhoun, 1971), and the parameter estimates used to quantify the pH dependence of binding kinetics.

RESULTS

Direct effects of pH_o on sodium channel inactivation

Before analyzing the effects of pH on LA interactions with the Na channel, it is important to consider the effects of pH alone. A considerable literature exists on H^+ modulation of Na channel gating and permeability in nerve (Hille, 1968; Drouin and The, 1969; Woodhull, 1973; Brodwick and Eaton, 1978; Courtney, 1979; Mozhayeva et al., 1984) and muscle (Nonner et al., 1980; Campbell and Hahin, 1984). There is general agreement that the sodium channel is directly blocked by external H^+, and that the proton binding site is a titratable group inside the pore, with pK_a in nerve ~5.4 (Woodhull, 1973). This places an effective lower pH limit on observations of pH_o dependence of LA block of sodium channels, because the signal (I_{Na}) is attenuated at low pH. As pH_o is made more alkaline, there is a hyperpolarizing shift in the voltage dependence of equilibrium Na channel inactivation, h_∞, which probably reflects binding of H^+ to negative surface charges (Hille, 1968). This effect of external H^+ on Na channel gating has been further characterized as a slowing of recovery from inactivation, τ_h, mediated by a selective decrease in the Hodgkin-Huxley rate parameter, α_h (Courtney, 1979).

Sufficiently slow recovery from inactivation at alkaline pH may interfere with the accurate measurement of LA dissociation during and following a pulse train, since there is no simple way to distinguish between an anesthetic-free inactivated channel and an anesthetic-bound channel. For this reason, control experiments were performed to assess changes in kinetics of recovery from inactivation (τ_h) in this preparation as pH_o is made alkaline. In four such experiments, there was a 5–10 mV hyperpolarizing voltage shift in h_∞ (mean = 7 mV) when the pH was raised from 7.3 to 10.4 (Fig. 1). The rate of recovery from inactivation, as a function of potential and pH_o, was

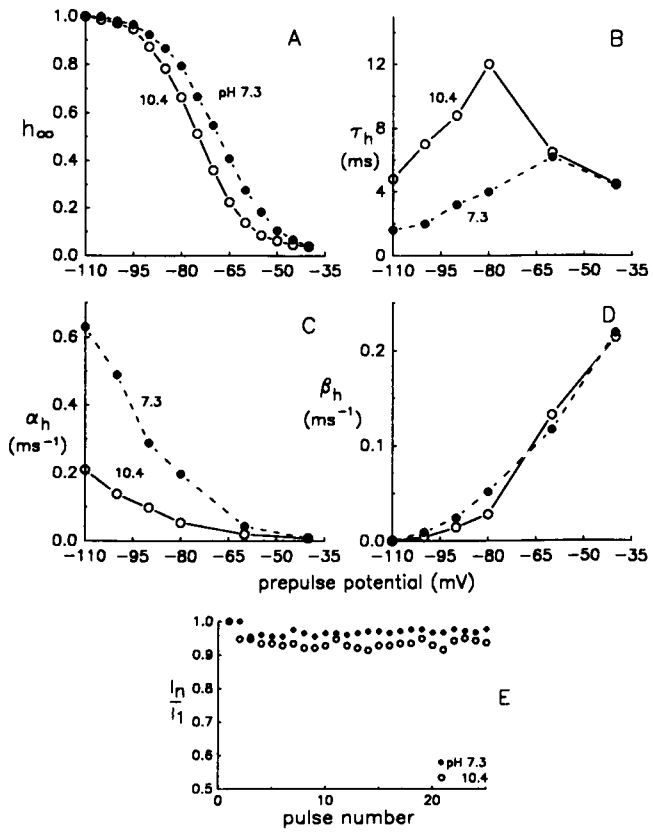


FIGURE 1 Effects of extracellular pH on Na inactivation. Raising pH from 7.3 to 10.4 produced a hyperpolarizing shift in the voltage-dependence of steady-state inactivation (*A*), and an increase in the time constant for recovery from inactivation, measured by a three-pulse protocol (*B*). The holding potential was −80 mV. The Hodgkin-Huxley rate constants for inactivation, α_h and β_h, were calculated from the data in *A* and *B*, and plotted against the prepulse potential (*C* and *D*). High pH produced large changes in α_h over the range of potentials tested (*C*), but had relatively little effect on β_h (*D*). Fiber 87N10B. In *E*, normalized peak I_{Na} is plotted against pulse number for 16-ms pulses to 0 mV at a rate of 10 Hz. There is not a significant change in the amount of decline in peak I_{Na} as pH_o is changed from 7.3 (●) to 10.4 (○). This data represents the largest decline in peak I_{Na} observed during pH controls at either pH; typical declines in the absence of drug were 1–2%. Fiber 87N04A.

assessed using a standard three-pulse protocol, consisting of a 50-ms conditioning pulse from the holding potential (−80 mV) to 0 mV (producing complete inactivation), followed by a variable duration prepulse to a given potential, then a fixed duration test pulse to 0 mV. Consistent with similar studies of inactivation kinetics (Chiu, 1977), I_{Na} recovery could be described as a single exponential, with a small (100–200 μs) delay (data not shown).

The effects of pH_o on the kinetics of inactivation are similar to those described in frog nerve (Courtney, 1979). Recovery from inactivation is slower at high pH_o, the largest difference occurring at the holding potential (Fig. 1 *B*). In four experiments, τ_h ranged from 4 to 9 ms at pH 7.3, and from 12 to 20 ms at pH 10.4. These changes in h_∞ and τ_h with pH_o arise from a selective effect of pH on the Hodgkin-Huxley rate parameter α_h (Fig. 1, *C* and *D*). Thus, Courtney's analysis of pH_o effects in frog myelinated nerve in the pH range 6.4–7.4 appears also to apply to toad nerve in an alkaline pH range. For the purpose of providing pH "controls" for LA binding kinetics, the most trenchant observation made here is that, even at the highest pH_o, recovery from "fast" sodium inactivation is essentially complete within 50 ms after a depolarizing pulse. Provided the dominant time constant for recovery from block is long relative to τ_h (true for all the drugs in this study), there is a clean kinetic separation between the (presumed) parallel processes of normal recovery from inactivation and recovery from phasic block (drug unbinding).

As an alternative test of sodium current recovery kinetics at high pH_o in the absence of drug, peak currents were measured during 10-Hz pulse trains at pH 7.3 and 10.4 (Fig. 1 *E*). There was little "droop" in the magnitude of peak I_{Na} during the pulse train at either pH in four experiments; the data shown in Fig. 1 *E* represent the largest droop observed at either pH. This result indicates that slow Na inactivation, which would manifest itself as a progressive decrease in peak I_{Na} during the pulse train, is not significant under these conditions.

pH_o dependence of phasic block

The anesthetic under test was applied at a concentration producing ~50% tonic block at pH 7.3 (see Table 1). After stabilization of the Na currents, pulse trains were applied at two to four different frequencies, and peak I_{Na} during each pulse was plotted against pulse number. The Courtney model was fitted to the observed decrease in peak I_{Na} during the pulse trains to estimate the kinetics of binding and unbinding of drug (Chernoff, 1990). The solution bathing the node was then changed to one at a different pH, maintaining the same extracellular concentration of drug, and the phasic block protocol was repeated. Normalized peak I_{Na} from pulse trains applied at 1, 4, and 10 Hz, at three levels of pH_o, are shown from experiments with 200 μM lidocaine (Fig. 2 *A*) or 25 μM *d*-bupivacaine (Fig. 2 *B*). The figure also lists the rates of binding and dissociation during depolarization ($a \cdot k$ and $a^* \cdot l$, respectively) and the recovery rate between pulses, λ_r, determined by the best fits to data at all stimulation frequencies.

For both lidocaine and bupivacaine, pH_o had major effects on the kinetics of binding and dissociation during and after pulse trains. Summary kinetic data for all five drugs examined are given in Table 2. There are several

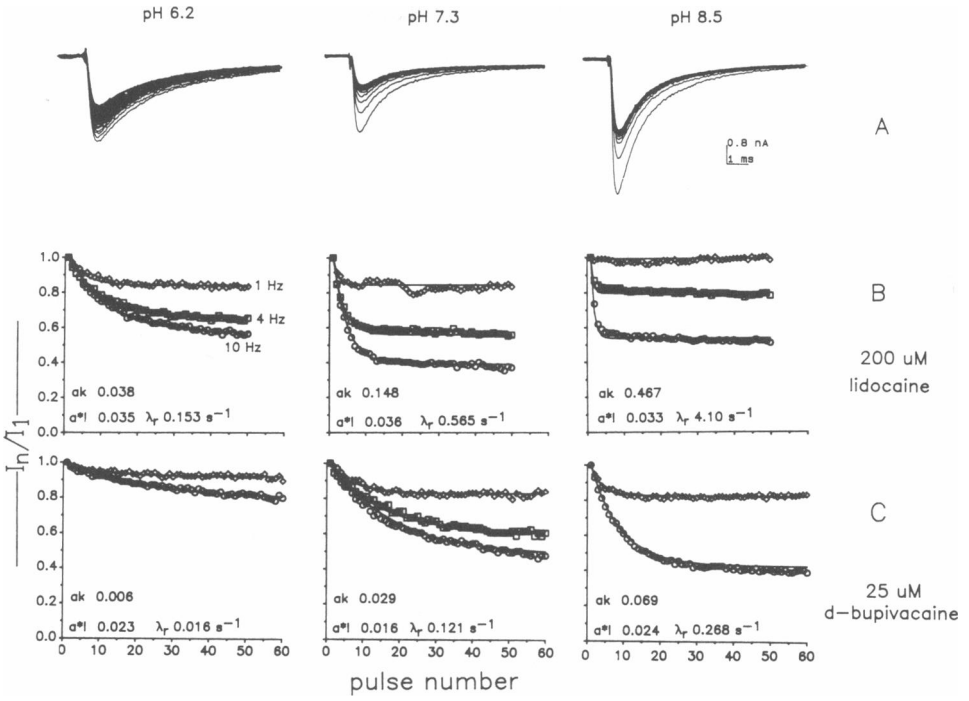


FIGURE 2 pH_o dependence of phasic block with lidocaine and *d*-bupivacaine. In *A*, selected raw current traces at each pH_o are shown for lidocaine (10 Hz stimulation rate). In *B* (lidocaine) and *C* (bupivacaine), the normalized peak currents are displayed for 10, 4, and 1 Hz stimulation rates. Lines drawn through the data points represent the best fit of the model, with parameter values shown inside each graph. The order of solution changes in these experiments was pH_o 7.3, then pH_o 8.5, then pH_o 6.2. The pH effects were usually reversible in this pH range; i.e., the block at pH_o 7.3 was similar before and after excursions to other levels of pH. Lidocaine data: fiber 87O19B; bupivacaine data: fiber 87821B.

trends which appear to hold for all the drugs. Examining the values of the parameters associated with binding during a pulse, $a \cdot k$ and $a^* \cdot l$, we note that $a \cdot k$ becomes large (i.e., more unbound channels become bound per pulse) at more alkaline pH_o. Interestingly, even the on-rate for the uncharged lidocaine homologue 5-HHX increases somewhat with alkaline pH_o, indicating that external protons may have a weak direct effect on the receptor. The dependence of $a^* \cdot l$ on pH_o is much weaker than that of $a \cdot k$, for all the agents tested. We suggest the following as possible explanations of these trends.

(a) As pH_o becomes more alkaline, a greater proportion of the extracellular drug exists as the uncharged form with partitions more strongly into the membrane. The membrane drug concentration rises, in turn raising the intracellular drug concentration of both charged and uncharged drug. Thus, increasing pH_o raises the drug concentration in all compartments thought to have access to the binding site. It is not surprising, then, that $a \cdot k$ (through the concentration dependence of k) increases under alkaline conditions. Changes in LA concentration, on the other hand, would not be expected to affect the (assumed) zero'th order unbinding rates contributing to $a^* \cdot l$.

(*b*) Extracellular H^+ may compete sterically with LA binding, or there may be electrostatic repulsion between H^+ and cationic LA. It is known that external Na^+ can antagonize block of Na channels by QX-314 (Cahalan and Almers, 1979), possibly by an interaction within the channel pore. External H^+ may antagonize block by a similar mechanism: although the H^+ activity in solution is about three to seven orders of magnitude lower than the Na^+ activity, H^+ ions are ~250-fold more permeant than Na^+ through nodal Na channels (Mozhayeva et al., 1984).

(*c*) Extracellular H^+ may affect LA binding allosterically. The phasic inhibition induced by the 16-ms-long pulses used in this study probably arise from LA binding to both open and inactivated channels (Chernoff, 1980). Elevation of pH_o increases the open channel probability through shifts in the voltage dependence of activation (Hille, 1968; Hille et al., 1975); however, neither the steady-state h_∞ nor the time constant for inctivation are altered by elevated pH for the test depolarization used here (Fig. 1). Therefore, the modulation by pH of phasic block with 5-HHX probably arises from an enhancement of the open (and possibly pre-open) state(s). The binding of ionizable LAs that combine strongly with the open

TABLE 2 pH dependence of phasic block kinetic parameters

Drug	Parameter	pH_o 6.2	7.3	8.5	9.4	10.4
	s^{-1}	($n = 3$)	($n = 9$)	($n = 5$)		
Lidocaine	λ_r	0.182 ± 0.029	0.651 ± 0.113	3.13 ± 1.42		
(200 μM)	$a \cdot k$	0.029 ± 0.008	0.093 ± 0.029	0.290 ± 0.080	ND	ND
	$a^* \cdot l$	0.042 ± 0.008	0.063 ± 0.021	0.070 ± 0.061		
		($n = 2$)	($n = 10$)	($n = 7$)		($n = 3$)
d-Bupivacaine	λ_r	0.056 ± 0.026	0.067 ± 0.031	0.626 ± 0.361		1.68 ± 0.070
(25 μM)	$a \cdot k$	0.015 ± 0.005	0.042 ± 0.024	0.075 ± 0.036	ND	0.088 ± 0.028
	$a^* \cdot l$	0.026 ± 0.005	0.032 ± 0.019	0.033 ± 0.016		0.023 ± 0.014
		($n = 3$)	($n = 5$)	($n = 2$)		
GX	λ_r	0.218 ± 0.052	0.471 ± 0.155	1.59 ± 0.660		
(800 μM)	$a \cdot k$	0.036 ± 0.011	0.050 ± 0.019	0.099 ± 0.029	ND	ND
	$a^* \cdot l$	0.095 ± 0.010	0.080 ± 0.020	0.055 ± 0.022		
			($n = 3$)	($n = 3$)		($n = 1$)
PRG-030	λ_r		0.038 ± 0.005	0.026 ± 0.008		0.085
(1 mM)	$a \cdot k$	ND	0.016 ± 0.004	0.060 ± 0.003	ND	0.151
	$a^* \cdot l$		0.015 ± 0.005	0.020 ± 0.003		0.030
		($n = 1$)	($n = 6$)	($n = 1$)	($n = 1$)	
5-HHX	λ_r	0.940	1.32 ± 0.31	1.32	1.96	
(2 mM)	$a \cdot k$	0.049	0.060 ± 0.011	0.076	0.141	ND
	$a^* \cdot l$	0.065	0.075 ± 0.011	0.100	0.021	

Parameters fit to data from pulse trains at three stimulation frequencies. Each pulse was 16 ms in duration and at a potential 0 mV. Holding potential was −80 mV. Values reported are means ± SE. Parameters $a \cdot k$ and $a^* \cdot l$ calculated from Eqs. 13 and 14 of Chernoff (1990). ND, not determined.

state will be influenced by this effect of protons as well as through drug ionization.

A second trend appears when comparing the rate constants among drugs of differing hydrophobicity. Among compounds with comparable solution pK_a's, e.g., lidocaine, bupivacaine, and GX, the *on* binding rates, $a \cdot k$, vary by only two- to threefold even though the solution concentrations of drug differ by 80-fold and the hydrophobicities (assessed by P°) range over three orders of magnitude. Dissociation rates from depolarized channels ($a^* \cdot l$) are almost independent of hydrophobicity, differing less than threefold at pH 7.3 for all the LAs except PRG-030.

Two possible explanations address these data. The first is that comparable *on* rates ($a \cdot k$) result from the product of vastly differing concentrations of drug in solution (which binds to a site directly accessible from the aqueous axoplasmic compartment) times inversely differing rate constants. For example, GX at 800 μM would bind with a rate constant about 30 times smaller than that of *d*-bupivacaine at 25 μM. The second explanation is that these drugs act from within the membrane (a "hydrophobic pathway," Hille, 1977*b*) wherein their free concentrations are modified by partitioning from the aqueous phase. However, quantitative estimates of the drugs' "membrane concentrations," equal to the product of the neutral species concentration in the bathing solution times P° (from Table 1; cf. Courtney, 1980*b*), range over four orders of magnitude, highly disproportionate to the relative rates of binding. Despite the lack of quantitative agreement, it is clear that less hydrophobic drugs (judged by P°), used at a correspondingly higher concentration in solution to yield comparable tonic block, block phasically at rates similar to those of more hydrophobic ones; this conclusion is elaborated in the Discussion. The constancy of the *off* rate, $a^* \cdot l$, among compounds of widely differing hydrophobicity, implies that neither the retention at the binding site by hydrophobic forces nor the diffusion through some "hydrophobic pathway" is rate limiting for drug dissociation from depolarized channels.

pH dependence of recovery from phasic block

We now focus our attention exclusively on the pH dependence of recovery from phasic block. For each drug, the recovery rate between pulses, λ_r is plotted as a function of pH_o (Fig. 3). For those drugs with solution pK_a's intermediate between pH 6.2 and 10.4 (lidocaine, bupivacaine, and GX), the rate of recovery is significantly modulated by pH_o, becoming faster as the pH was increased. For the two drugs whose pK_a's lie outside the range of pH tested (PRG-030, 5-HHX), there is relatively little effect of pH on λ_r. We conclude that external pH mainly affects the anesthetic and not the dissociation

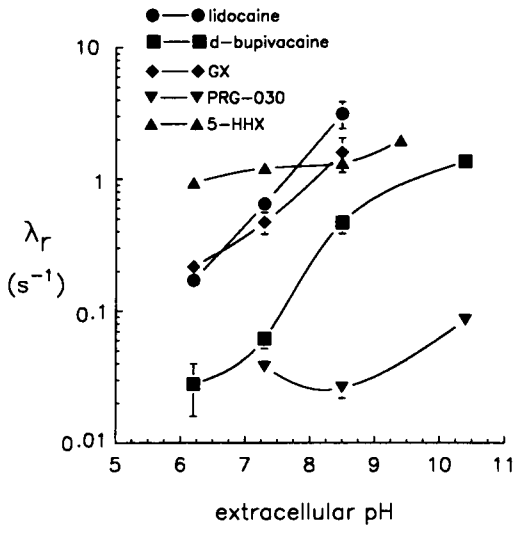


FIGURE 3 pH dependence of the rate of recovery from phasic block (λ_r). Extracellular pH changes produced large changes in recovery rate for those drugs (lidocaine, bupivacaine, GX) which have pK_a's within the pH range tested (note logarithmic scales). The pH_o dependence of recovery rate for 5-HHX (predominantly neutral in this pH range) and PRG-030 (predominantly charged) were small compared to that observed with the protonatable drugs. Error bars indicate standard errors where appropriate. The curves connecting the data points for each drug are arbitrary. Data from Table 2.

route for closed channels. These results are consistent with the model of Schwarz et al. (1977), i.e., titration of the tertiary amine occurs while the drug is bound to its receptor. Furthermore, there does not appear to be a common, limiting rate of recovery at high pH_o (favoring the neutral species) or at low pH_o (favoring the charged species). Therefore, there must be factors other than the extent of drug ionization that are important in determining the rate of recovery from phasic block. These considerations are examined in greater detail below.

Modeling recovery: deprotonation and dissociation rates

As Hille (1977*b*) has postulated, there may be both "hydrophilic" and "hydrophobic" routes to a LA binding site located in or near the sodium channel. If we accept Hille's assumption that the hydrophilic route is only available when the channel is open, then how do charged local anesthetics depart from closed channels? For 3° amine LAs, deprotonation (and subsequent escape of the neutral species via the hydrophobic route) may be the dominant kinetic pathway (Schwarz et al., 1977; Yeh and Tanguy, 1985). An alternative possibility is that the drug escapes from the channel during the brief, random openings of Na channels ("flickering") that are thought to occur at rest (Starmer and Courtney, 1986); this pathway would be most important for the 4° amine LAs (which cannot lose their charge), and for those 3° amine LAs which escape only slowly via the hydrophobic route. A third possibility, favored by the analysis to follow, is that the charged form can depart directly via the hydrophobic route.

In contrast to the strong voltage dependence of the rate of recovery of sodium channels from inactivation, holding potential in this preparation has little effect on the rate of recovery from phasic block (Chernoff, 1990) with the caveat that this observation was made only for tertiary amine LAs at pH 7.3. We therefore postulate that the rate of recovery from phasic block is limited by the rate at which LA unbinds from Na channels, and not by the rate of recovery from inactivation. A three-state, four-parameter model for the unbinding of anesthetic from sodium channels is shown in Fig. 4 (although the unbinding step is indicated to be from the resting state in the figure, unbinding from an inactivated state is equally plausible; see Chernoff, 1990). This figure depicts dissociation of drug proceeding along two parallel pathways: (*a*) direct dissociation of charged drug (rate constant l_c), or (*b*) deprotonation (rate constant l_p), followed by dissociation of the neutral species (rate constant l_n). The forward rate constants for binding are assumed to be negligible, and l_p is related to the effective pK_a of the drug at the

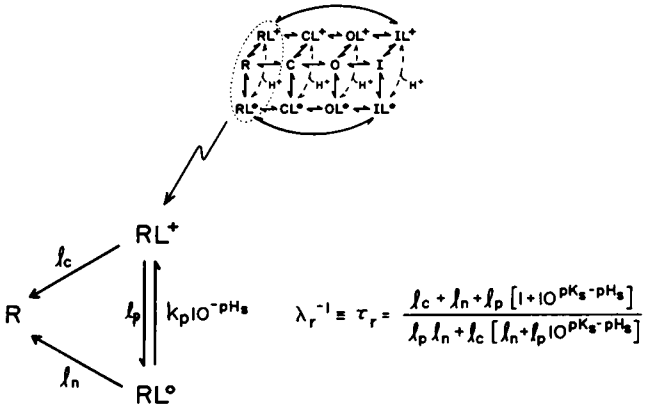


FIGURE 4 Kinetic model for unbinding of local anesthetic from closed Na channels. Dissociation of drug and/or deprotonation of the cationic form are assumed to be rate-limiting for recovery of functional Na channels. The rate constants l_c and l_n describe the dissociation rates of the charged and neutral forms of anesthetic; the rate constants k_p and l_p desribe the forward and reverse rate constants, respectively, for protonation of the bound anesthetic; the equilibrium constants pK_s and pH_s describe the effective pK_a of the bound drug and the effective pH in the vicinity of the titratable moiety. Binding of drug to the receptor under these conditions is assumed to be minimal. The dominant time constant for this closed reaction loop is given by the equation to the right of the diagram.

binding site, pK_s, and the forward protonation rate constant, k_p, by $l_p = k_p 10^{-pK_s}$. This model has a dominant relaxation rate, λ_r, given by

$$\lambda_r = \tau_r^{-1} = \frac{l_p \cdot l_n + l_c[l_n + l_p 10^{(pK_s - pH_s)}]}{l_c + l_n + l_p(1 + 10^{(pK_s - pH_s)})}. \quad (1)$$

A derivation of this equation can be found in Starmer and Courtney (1986). An alternative model, used in most other studies of pH-dependent recovery kinetics (e.g., Moorman et al., 1986; Schwarz et al., 1977), does not permit direct dissociation of the charged species. The relaxation rate under these conditions (trapping of cation) can be derived from Eq. 1 by setting l_c to zero:

$$\lambda_r = \frac{l_p \cdot l_n}{l_n + l_p[1 + 10^{(pK_s - pH_s)}]}. \quad (2)$$

These two versions of the three-state recovery model (Fig. 4) were used to fit the experimentally determined recovery kinetics for lidocaine and bupivacaine from Fig. 3. The fitting procedure was by trial and error, using as a starting point the solution pK_a of each drug and a (diffusion limited) protonation rate constant, k_p, of 10^9 $M^{-1}s^{-1}$. The pH at the binding site, pH_s, was arbitrarily set equal to the solution pH; note that, because pK_s and pH_s always appear together in Eqs. 1 and 2, no mathematical procedure can estimate each parameter uniquely.

Applying this parameter estimation procedure, reasonably good fits of the model allowing dissociation of the charged species to the lidocaine and bupivacaine recovery kinetics were obtained (Fig. 5 *A*, *solid lines*). The "trapped cation" model (Eq. 2), however, underestimates the recovery rates at pH 6.2 (*dashed lines*). A similar conclusion can be drawn by fitting these models to data from previous studies of pH-dependent recovery kinetics (Fig. 5, *B* and *C*): in each case, the trapped cation model underestimates the recovery rate for pH $\ll pK_a$. Hence, dissociation of the charged species appears to contribute to the overall recovery rate in all three studies, and becomes the dominant kinetic pathway for pH $\ll pK_a$. Further support for this conclusion follows from the relatively constant value of λ_r for PRG-030 over the pH range 7.3–10.4 (Fig. 3 and Table 2), despite a dwell time in the neutral form which depends exponentially on pH in this range.

The parameter estimates used in the previous figure for each model are given in Table 3. Although these estimates are not unique, as discussed above, they form a basis for comparison between drugs and between studies. For example, for each LA the apparent pK_a of bound drug (given pH_s equal to pH_o) is slightly higher than the pK_a in free solution, the largest difference occurring with W6211 (pK_s 7.2, pK_a 6.3). Second, the parameter estimates for

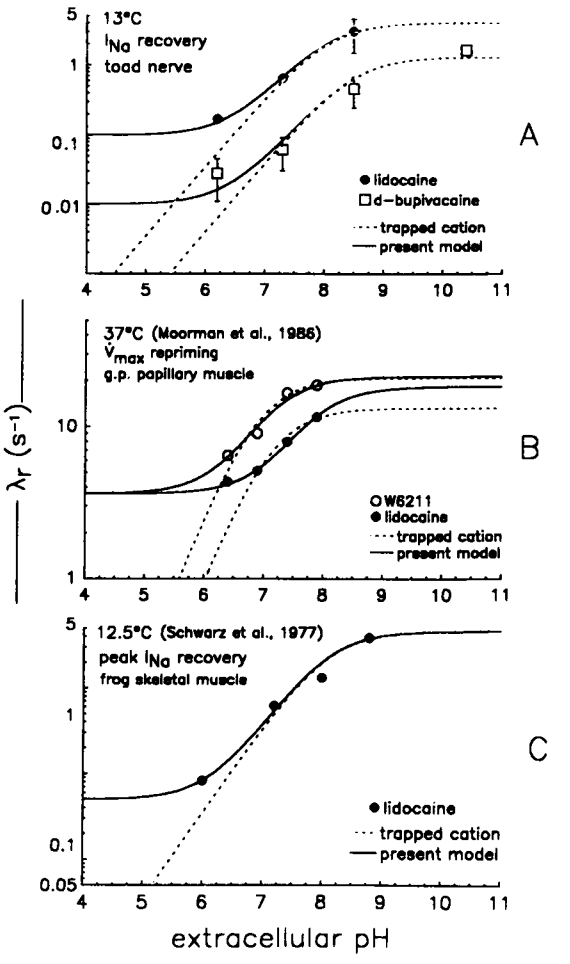


FIGURE 5 Fits of the recovery model (Fig. 4) to the pH dependence of the rate of recovery from phasic block in this study (*A*) and the data of Moorman et al. (1986) (*B*) and Schwarz et al. (1977) (*C*). For the data from each study, the more general model, including an unbinding rate for the cationic species (*solid lines*), fits the data at low pH better than a trapped cation model (*dashed lines*). The best fits for either model predict a proton dissociation constant for bound drug 0.2–0.7 pH units higher than in free solution (parameter estimates in Table 3). Dissociation rates for the cation are 10- to 200-fold slower than for the neutral form.

the lidocaine data from this study and from Schwarz et al. (1977) are comparable, whereas the rate constant estimates from the data of Moorman et al. (1986) are much higher, particularly for dissociation of the charged species (360-fold higher l_c). We speculate that these faster recovery kinetics are related to the higher temperature at which that study was performed although tissue specificity, and the difference in method used to obtain unbinding data ($[dV/dt]_{max}$ rather than peak I_{Na}), may also be involved.

TABLE 3 **Estimates of dissociation and protonation rate constants from recovery data**

Drug	k_p	l_p	l_n	l_c	pK_s (binding site)	Temperature	Data source
	$M^{-1}s^{-1}$	s^{-1}	s^{-1}	s^{-1}		°C	
lido	$2 \cdot 10^9$	10	7	0.1	8.3	13	this study
d-bupiv	$2 \cdot 10^9$	4	2	0.01	8.7		
lido	$4.8 \cdot 10^9$	38	33	3.62	8.1	37	Moorman et al. (1986)*
W6211	$3.2 \cdot 10^9$	200	24	3.62	7.2		
lido	$1.6 \cdot 10^9$	10	5.5	0.05	8.2	12.5	Schwarz et al (1977)‡

pH_s (binding site) assumed equal to pH_o. *From [dV/dt] repriming data, guinea pig papillary muscle. ‡From phasic block of I_{Na}, frog skeletal muscle; recovery rates obtained by applying simplified kinetic model to data from Fig. 9 of Schwartz et al. (1977).

Molecular weight dependence of recovery rates: neutral vs. charged agents

Factors other than charge regulate the rate of recovery from inhibition in repolarized membranes. Following a proposal by Courtney (1984), we examine the dependence of λ_r on molecular size in Fig. 6, including data from this study and others using ionizable and permanently charged or uncharged LAs. With the exception of lidocaine, the neutral species dissociate at a rate of 1–2 s^{-1}, independent of molecular weight. Lidocaine is unique among these compounds in its ability to bond intramolecularly when in the neutral (base) form, a tendency greatly amplified in low dielectric solvents (McMaster, P. D., V. J. Noris, C. E. Standard, E. W. Byrnes, and P. R. Guzzo, submitted for publication), and this behavior may account for its faster dissociation rate. In contrast, molecular weight exerts a marked effect on the dissociation of charged LAs, be they protonated 3° amines or permanently charged 4° amines. Lidocaine homologs weighing <240 D leave the binding site four to five times faster than those weighing >260 D. There is no correlation of λ_r with hydrophobicity, for either neutral or cationic LAs. The apparent discrimination by molecular weight for cationic drug dissociation implies that the rate-limiting step is sensitive to the size of the ligand, a sensitivity not shared with the slowest step for dissociation of the neutral species.

A value for a nonhomologous "anesthetic," 9-amino acridine (9-AA), is also plotted in Fig. 6. The recovery rate for this cationic drug, which produces a strong phasic inhibition of I_{Na}, lies well off the curve for the lidocaine homologs. To us, this emphasizes the importance of comparing structurally related compounds when examining phenomena like use-dependent block which can arise through a variety of mechanisms involving different channel states and transitions (Cahalan et al., 1980).

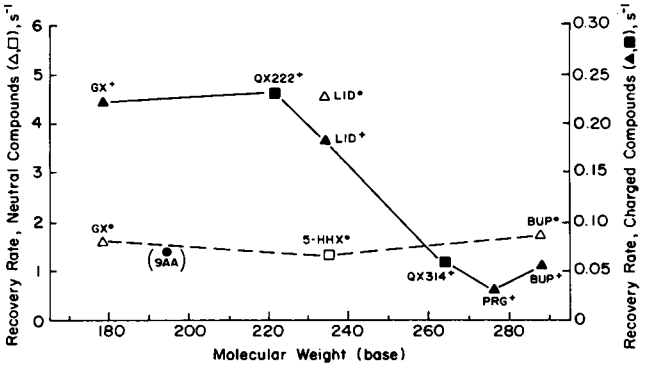


FIGURE 6 Rates of recovery from inhibition during the interpulse (λ_r) as a function of LA molecular mass. Values for neutral compounds (*open symbols*) are from this paper, with rates for 3° amine LAs approximated by those at the highest pH_o (cf. Table 2). Values for charged compounds are either from this paper (3° amines at the limiting rate at low pH_o) or from Yeh and Tanguy (1985; 4° amines and 9AA at neutral to slightly alkaline pH_o).

DISCUSSION

General conclusions and comparison with previous studies

In this study, we have investigated the pH dependence of phasic block with five local anesthetics, paired either by pK_a or by lipid solubility of the neutral species. We have estimated the *on* and *off* rates for inhibition of Na current during a depolarizing pulse, and the rate of recovery from this block between pulses. Our results show that, in general, alkaline pH potentiates the binding of anesthetic during a pulse, but simultaneously accelerates the rate of recovery between pulses; the second finding is consistent with similar studies performed previously in nerve and muscle (Khodorov et al., 1976; Schwarz et al., 1977; Grant et al., 1982; Broughton et al., 1984; Moorman et al., 1986). The net effect of pH_o on phasic block thus

depends critically on the frequency of stimulation (Fig. 3), which determines the relative contributions of net binding and dissociation to steady-state phasic block.

Is steady-state phasic block (b_{ss}) an adequate measure of anesthetic potency?

Steady-state phasic block, using a single stimulation frequency, is sometimes taken as a measure of anesthetic potency, but the results shown here call into question the general validity of that measure. For the data in Fig. 2, for example, the increase in total anesthetic "uptake" per pulse at alkaline pH, coupled with the faster recovery from block between pulses, had partially offsetting effects on b_{ss} (see Eq. 16, Chernoff, 1990). Depending on whether the stimulation frequency was fast or slow relative to the recovery rates, changes in pH_o resulted in large (e.g., lidocaine at 1 Hz) or small (e.g., lidocaine at 10 Hz) changes in b_{ss}. Therefore, b_{ss} alone yields relatively little information regarding the kinetics of phasic block or anesthetic potency. Analysis of use-dependent drug actions based on the steady-state level of phasic block (e.g., Yeh and ten Eick, 1987) provide only limited information on which to base molecular mechanisms.

Role of extracellular H^+ on LA binding

Quantitative analysis of the pH dependence of recovery from block (Figs. 3 and 4) reveals a nonnegligible role for direct dissociation of the charged form of LA from the Na channel at rest; this reaction becomes rate limiting for $pH_o \ll pK_a$ (Fig. 5). Furthermore, the apparent pK_a of drug when bound to its receptor appears to be quite close to the pK_a in free solution (but see below). This conclusion differs considerably from that drawn by Moorman et al. (1986), who found that "pK_a does not predict pH potentiation of sodium channel block." However, their analysis appears flawed by two contradictory assumptions: first, that the free solution pK_a, together with the external pH, determines the ratio of charged to neutral drug on the receptor; second, that the apparent pK_a of the bound drug (pK_s) determines the rate at which it deprotonates. When these contradictory assumptions are replaced by one in which both the charged/neutral ratio and the deprotonation rate are set by pK_s, their data supports our conclusion that apparent $pK_s \approx pK_a$. Thus, Courtney's (1987) use of free solution pK_a as a component of a quantitative structure–activity relationship appears justified.

Interpretations of the role of pH on LA actions have major ramifications on the validity of quantitative modeling. Although previous treatments of LA ionization have implicitly assumed rapid protonation and consequent equilibria throughout the binding reaction, the actual rates of protonation–deprotonation reactions for bound LAs as well as for those "free" molecules in the solution or in the membrane may be as slow as the rates of drug binding to the channel. For example, Schwarz et al. assumed a diffusion-limited value for the solution protonation *on* rate (k_{on}^{H+}) of lidocaine of $2 \cdot 10^9\ M^{-1}s^{-1}$; the corresponding *off* rate (k_{off}^{H+}), is $12.6\ s^{-1}$ (for $K_a = 0.5 \cdot 10^{-8}$ M; $pK_a = 8.4$ at 13°C), and the protonation relaxation time at pH 7.3 ($\tau_{7.3}$) is 9 ms. This value is comparable with that of channel open lifetimes and to the time constants for LA binding during depolarization (cf. Table 2). If the faster bimolecular protonation rate constant of $2.5 \cdot 10^{10}\ M^{-1}s^{-1}$ is used (Eigen and Hammes, 1963), then $\tau_{7.3}$ falls, but only to 2 ms.

In a kinetic scheme where LA molecules in solution are binding to a directly yet transiently accessible site (e.g., "open channel block"), the protonation *kinetics* as well as the equilibrium value (pK_a) will affect the overall binding of drug. In schemes that involve direct binding of LA from the membrane phase, the role of protons differs. Selective access to the receptor of the neutral drug molecules (i.e., membrane partitioning, the hydrophobic pathway) constrains the role of protons to the regulation of the concentration of drug in the hydrophobic phase. Even in the case where ionized as well as neutral species may reside on the membrane, drug protonation may be far slower than in solution, so ionization remains at steady state during the binding reaction.

Experiments show that LAs bound to neutral phospholipid bilayer membranes have effective pK_a's about one pH unit below the solution pK_a (Boulanger et al., 1981; Schreier et al., 1984). This equilibrium relation is identical to having a 10:1 ratio of membrane/buffer partition coefficients for the neutral and ionized species of LA, respectively. Selective binding to membranes, even in a relatively nonhydrophobic region (Watts and Poile, 1986), therefore, most also change the rates of proton binding and/or dissociation. The ionization behavior of LA bound to the sodium channel may range between the extremes of solution and the membrane interior. Bound drug molecules may have almost free access to external protons, as Schwarz et al. (1977) have proposed. Alternatively, a large difference between H^+ activity at the LA binding site and the solution pH may, fortuitously, equal a large change in pK_a of the drug upon binding.

Dissociation under acid conditions: entry into bilayer vs. channel flickering

The data and modeling presented here suggest that direct dissociation of charged drug from the LA binding site

between depolarizations is significant under acid conditions. The charged drug may escape via lateral dissociation into the bilayer, consistent with recent observation of significant uptake of protonated LAs into biological membranes (Boulanger et al., 1981; Watts and Poile, 1986; Sanchez et al., 1988). (Membrane/buffer partition coefficients for tetracaine are 10^4 and 3×10^3, respectively, for neutral and protonated species, whereas the same parameters for octanol/buffer are 3×10^4 and 4.0, i.e., four orders of magnitude difference. Evidently, there are important charge interactions in the membrane that are not reproduced in the solvent system.) One must also consider the possibility that this rate constant represents the frequency with which anesthetic-bound Na channels flicker open to release the trapped cation. This possibility was investigated in detail by Yeh and Tanguy (1985), who measured the rate of recovery from phasic block, as a function of holding potential, with lidocaine and its triethyl (QX-314) and trimethyl (QX-222) quaternary homologs. They found little voltage dependence of recovery rate with lidocaine, but recovery with the quaternary drugs was strongly voltage dependent, becoming slower (*e*-fold per 14 mV) when the membrane was hyperpolarized. Also, at a given potential, recovery from QX-314 block was four to five times slower than that from block by QX-222. Yeh and Tanguy accounted for these findings in the following way: (*a*) The rate-limiting step in recovery from block with QX-222 or QX-314 is channel opening, because these drugs cannot (it is assumed) use the hydrophobic pathway; therefore, hyperpolarization (by decreasing the frequency of channel openings) slows the recovery rate. (*b*) The rate-limiting step in dissociation of lidocaine is deprotonation (assumed to be a voltage-independent process). (*c*) The difference in the rate of recovery from block with QX-222 and with QX-314 at a given potential is due to their unequal size, i.e., the smaller molecule, QX-222, escapes from the channel more rapidly.

Our results agree with the size-dependent postulate of Yeh and Tanguy (*c*), but we maintain that cationic drugs may escape into the membrane, not just into aqueous solution through the channel pore. In keeping with the size dependence of recovery (Fig. 6), charged LAs of <240 D squeeze through this pathway about five times faster than those >250 D. This notion is consonant with Courtney's (1984) data and his concept of a narrow passage for LA escape. The lack of correlation between escape rate and hydrophobicity implies that neither lipid solubility nor hydrophobic absorption is a unique determinant of drug passage by this route.

The assignment of the rate-limiting step of lidocaine dissociation to drug protonation (*b*) requires that the dissociation rate of the charged form be slow relative to the frequency of channel openings and the rate of deprotonation. Our data suggest that these conditions depend on the specifics of the preparation and the drug. PRG-030, for example, appears to dissociate mainly as the charged species at pH 7.3 and 8.5, where the charged species is favored (Fig. 3 and Table 2). Furthermore, the voltage dependence of the rate of recovery from lidocaine and bupivacaine block is almost absent in node (Chernoff, 1990), unlike the results reported in squid giant axon, although the voltage dependence of gating is similar in the two preparations.

Hydrophobicity and LA binding rates

Quite similar binding rates, $a \cdot k$ (Table 2), are obtained with LAs whose solution concentrations differed 80-fold. As a trend, the more hydrophobic LAs are more potent tonic blockers. We sought to account for the larger apparent rate constants, $a \cdot k/[L]$, of the more hydrophobic drugs by their higher concentration in the membrane, $[L_m]$. Using the approach of Courtney (1980*b*), we calculated $[L_m]$ as the product of P^o and $[L_s^o]$, the neutral species in solution, in turn calculated from the total LA concentration and the pK_a (from Table 1). The rate constants derived from this approach, $k_{on} = a \cdot k/P^o \cdot [L_s^o]$, also varied widely; e.g., 19-fold between GX and lidocaine. However, octanol provides a poor model for LA adsorption by phospholipid membranes (Watts and Poile, 1986; Sanchez et al., 1988), and the true membrane concentrations (yet unknown) may correlate more closely with the binding rates.

Another complexity in this analysis originates in the contribution of several channel states to the net binding that occurs during a 16-ms depolarization. The preceding paper showed that over this time lidocaine binds to inactivated, open, and possibly pre-open states (Chernoff, 1990). We have previously reported that the dependence of phasic block on pulse duration varies among different LAs, probably because of the different relative of state-dependent binding (Chernoff and Strichartz, 1989*b*). A study with shorter pulses would selectively elicit binding to the open (and pre-open) state, with less contribution from the inactivated state. More meaningful structure-activity relationships will perhaps be elicited by such careful deconvolution of net binding affinity into affinities for individual channel states.

The influence of external pH on channel-modifying drugs bound to their receptor may apply to systems other than Na channels and local anesthetics. Recent studies investigating the pH dependence of calcium channel block by dihydropyridines (DHP: Kass et al., 1988) show remarkable parallels to LA block of Na channels. These authors demonstrate that raising pH_o from 7.4 to 10.0 dramatically accelerates recovery of I_{Ca} from voltage-dependent block by the DHP amlodipine (pK_a 8.6). It

remains to be seen whether the mechanisms of block, and the routes of access to their respective receptors, are similar for the dihydropyridines and local anesthetics, but it appears likely that interactions of external H^+ with protonatable drugs acting within the cell membrane have implications for structural modeling and pharmacology extending beyond the scope of local anesthetics and Na channels.

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