Kinetic analysis of phasic inhibition of neuronal sodium currents by lidocaine and bupivacaine

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ABSTRACT Phasic ("use-dependent") inhibition of sodium currents by the tertiary amine local anesthetics, lidocaine and bupivacaine, was observed in voltage-clamped node of Ranvier of the toad, *Bufo marinus*. Local anesthetics were assumed to inhibit sodium channels through occupation of a binding site with 1:1 stoichiometry. A three-parameter empirical model for state-dependent anesthetic binding to the Na channel is presented: this model includes two discrete parameters that represent the time integrals of binding and unbinding reactions during a depolarizing pulse, and one continuous parameter that represents the rate of unbinding of drug between pulses. The change in magnitude of peak sodium current during a train of depolarizing pulses to 0 mV was used as an assay of the extent of anesthetic binding at discrete intervals; estimates of model parameters were made by applying a nonlinear least-squares algorithm to the inhibition of currents obtained at two or more depolarizing pulse rates. Increasing the concentration of drug increased the rate of binding but had little or no effect on unbinding, as expected for a simple bimolecular reaction. The dependence of the model parameters on pulse duration was assessed for both drugs: as the duration of depolarizing pulses was increased, the fraction of channels binding drug during each pulse became significantly larger, whereas the fraction of occupied channels unbinding drug remained relatively constant. The rate of recovery from block between pulses was unaffected by pulse duration or magnitude. The separate contributions of open (O) and inactivated (I) channel binding of drug to the net increase in block per pulse were assessed at 0 mV: for lidocaine, the forward binding rate k_O was $1.4 \cdot 10^5\ M^{-1}s^{-1}$, k_I was $2.4 \cdot 10^4\ M^{-1}s^{-1}$; for bupivacaine, k_O was $2.5 \cdot 10^5\ M^{-1}s^{-1}$, k_I was $4.4 \cdot 10^4\ M^{-1}s^{-1}$. These binding rates were similar to those derived from time-dependent block of maintained Na currents in nodes where inactivation was incomplete due to treatment with chloramine-T. The dependence of model parameters on the potential between pulses (holding potential) was examined. All three parameters were found to be nearly independent of holding potential from -70 to -100 mV. These results are discussed with respect to established models of dynamic local anesthetic-Na channel interactions.

INTRODUCTION

The primary pharmacological target of local anesthetics (LA) is the voltage-dependent sodium channel (Taylor, 1959; Hille, 1966). LAs reduce the sodium permeability of excitable membrane by inhibiting current flow through sodium channels; the mechanism may involve both occlusion of the pore and alteration of the gating properties of the channel (Cahalan and Almers, 1979*a*, *b*; Khodorov, 1981; Neumcke et al., 1981; Bekkers et al., 1984; Dubois and Schneider, 1985). Seminal studies of voltage- and frequency-dependent ("phasic") block of Na currents by LAs (Strichartz, 1973; Courtney, 1975; Khodorov et al., 1976; Hille, 1977*a*, *b*; Schwarz et al., 1977) have led to the use of LAs as molecular probes of Na channel structure and function and have provided insight into the mechanisms underlying the antiarrhythmic properties of these agents.

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Two alternative kinetic models of LA inhibition of Na currents have emerged from analysis of "phasic inhibition" of Na currents (reduction in I_{Na} by repetitive depolarizations): these are known as the modulated receptor hypothesis (MRH; Hille, 1977*b*; Hondeghem and Katzung, 1977) and the guarded receptor hypothesis (GRH; Starmer et al., 1984). Both hypotheses assume that (*a*) binding of an anesthetic molecule to a receptor obeys first-order reaction kinetics; (*b*) unbinding of anesthetic obeys zero'th-order kinetics; (*c*) anesthetic-bound channels are nonconducting; and (*d*) the forward and reverse binding rates are dependent on the state of the Na channel, as defined by Hodgkin-Huxley or other gating formalism. The two hypotheses diverge mainly with regard to the physical mechanism(s) controlling the effective forward and reverse rate constants. Under the MRH, the receptor affinity for LA changes with the channel state (e.g., by modulation of the off-rate), and is higher for open and inactivated states ("tighter" binding) than for resting states (Courtney, 1975). This presumably results from state-dependent changes in the configuration

of the binding site itself. Under the GRH, the receptor affinity is constant but access to the receptor is "guarded" by portions of the channel protein, e.g., the gating structures regulating the flow of ionic current. Both models may be considered special cases of a general state-dependent binding schema, in which LA (possibly in both charged and neutral forms) reacts with every defined channel state with unique rate constants (Fig. 1).

Further progress in refining our understanding of the LA:Na channel interaction has been limited by the absence of robust techniques to estimate the rate constants for LA binding to each Na channel state. There are at least two measurement problems: (*a*) The best available assay for binding is the loss of Na channel function, but this can only be assessed over a limited range of membrane potential, and, moreover, requires perturbing the system through membrane depolarization to measure the availability of functioning channels; (*b*) there is a continual admixture of states in the population of channels being studied, making it difficult to assess separately the rate of LA binding to each state. Despite these problems, various techniques have been developed to extract the binding rate constants and/or anesthetic affinities of identifiable Na channel states from electrophysiologic measurements (see, e.g., Hondeghem and Katzung, 1977; Schwarz et al., 1977; Starmer et al., 1984).

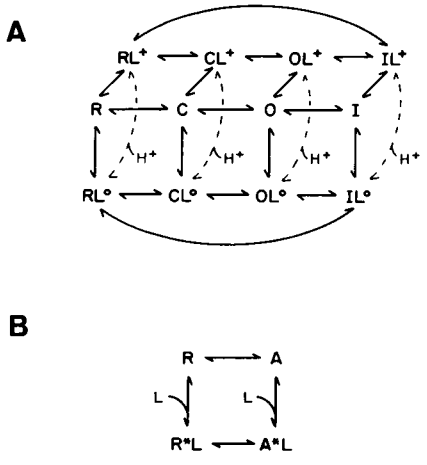

FIGURE 1 Kinetic schemes for local anesthetic binding to sodium channels. (*A*) General scheme for binding of tertiary amine drugs. Sodium channels are depicted as existing in resting (*R*), partially activated (*C*), open (*O*), and inactivated (*I*) states. Each state can bind anesthetic in either the neutral (L^*) or protonated (L^+) form. The rates of binding and equilibrium binding will, in general, depend on the channel conformation as well as the concentration (more properly, activity) of each form of drug in compartments having access to the binding site. Bound drug can gain or lose a proton (*dashed arrows*) by exchange with the extracellular compartment, but with rate constant and pK_a that may differ from those measured in bulk solution. Only state *O* is able to conduct sodium current. (*B*) Reduced kinetic model. Sodium channel conformations are lumped into a resting state (*R*) and a single activated state (*A*) which encompasses all states through which the channel passes when the membrane is depolarized. Drug binding to the resting state is weak, either due to the low affinity of this state or guarded access to the binding site. Drug binding to the activated state is strong, due to a higher affinity and/or unguarding of the binding site. For simplicity, no distinction is made here between charged and neutral forms of drug.

One such estimation procedure, relying entirely on observations of phasic (use-dependent) block of Na currents, was described early by Courtney (1975), and later refined by Courtney et al. (1978). This is a four-state, composite discrete/continuous "lumped" model, developed to estimate the apparent rate constants in a modulated receptor scheme. Their approach, as originally described, did not permit determination of the "true" binding rates, but did allow for quantitative estimates of (*a*) the fractional increase in blocked channels produced by each pulse in a depolarizing pulse train, and (*b*) the rate of recovery from use-dependent block between pulses. An equivalent method has been used to model ion channel blockade under a guarded receptor hypothesis (Starmer and Courtney, 1986).

An apparent limitation of the Courtney model, caused by lumping of states, is the loss of information on the detailed time course of binding and unbinding of LA during each pulse: the parameter estimates obtained are weighted time-integrals of the true (instantaneous) binding and unbinding rates; these instantaneous rates are presumably time-varying, because channels pass through multiple transient states (each with a different LA affinity and/or accessibility; see Fig. 1) during and after a depolarizing pulse. Therefore, there is not a simple relationship between the model parameters (derived from pulses of fixed duration and amplitude) and the more fundamental rate constants that govern the association of LA with these transient channel states. This limitation is addressed in the present study.

Here, I show that Courtney's four-state model *can* be used to obtain detailed information on the time course and voltage dependence of binding and unbinding of LA during a depolarizing pulse train. The model is re-derived from the general case of multiple LA-bound states. In this model, all possible binding reactions during a pulse are lumped into two terms: one ($a \cdot k$) describes the fraction of drug-free channels that bind LA during the pulse; the other ($a^* \cdot l$) describes the fraction of previously-bound channels that unbind LA during the pulse. Recovery from block (unbinding of LA) between pulses is described by a single rate parameter (λ_r), lumping the contributions from all recovery pathways together. A novel, robust parameter estimation procedure is developed, based solely

on measurements of the decrease in peak I_{Na} during a train of repetitive depolarizations of nerve membrane held in voltage-clamp. By calculating values for $a \cdot k$ and $a^* \cdot l$ over a range of pulse durations, the model is used to follow the time course of binding and unbinding of LA during a depolarizing pulse. Estimates of the forward rate constants for binding of lidocaine and *d*-bupivacaine to open and to inactivated channels at a membrane potential of 0 mV are obtained by calculating the integrated availability of those two channel states (a_O and a_I) as a function of time; these estimates are compared to those made by observation of time-dependent inhibition of noninactivating sodium currents following chloramine-T treatment. In the next paper (Chernoff and Strichartz, 1990), similar analytical techniques are used to assess the dependence of anesthetic binding and unbinding rates on drug charge and hydrophobicity.

MATERIALS AND METHODS

Myelinated nerve voltage clamp

Single myelinated nerve fibers from sciatic nerves of the toad (*Bufo marinus*) were isolated, mounted in a Lucite chamber, and voltage clamped at 13°C as described by Dodge and Frankenhaueser (1958). Salt bridges and calomel electrodes, filled with 1 M potassium chloride (KCl), were used to connect the electronics to the voltage clamp chamber. A larger than usual (20 mV) depolarizing offset within the clamp circuit (probably representing the sum of junction potentials) was detected after but was present throughout the experiments described here; all potentials reported here have been corrected for this offset. The voltage-dependence of gating parameters (e.g., steady-state inactivation) is comparable to those reported for other anuran nodes of Ranvier in similar conditions (Strichartz, 1973; Courtney, 1975; Hille, 1977*a*, *b*). The shunting artifact of Dodge and Frankenhaueser (1958) was not measured or adjusted for. The effective bandwidth of the clamp circuit ranged from 15 to 30 kHz. Voltage control during activation was improved by the use of series resistance compensation. The linear leakage and capacitance currents were subtracted by an analog circuit with two exponential time constants. This circuit was adjusted every few minutes during an experiment to null the membrane response to a small (10–20 mV) hyperpolarizing pulse. For experiments using chloramine-T, leak currents were further compensated by a -P/5 pulse procedure or by repeating the depolarizations after blocking Na currents with 0.1 μM tetrodotoxin. The holding potential was normally set at −80mV (corrected potential) to remove most resting fast sodium channel inactivation and minimize "ultra-slow" inactivation (Fox, 1976). The volume of the solution bathing the node of Ranvier was 0.1 ml, and the length of axon in this pool was 150 μm. About 2 ml of test solution was exchanged through this pool for drug introduction and removal.

Voltage clamp "command" pulses were generated by computer software (pCLAMP, Axon Instruments Inc., Burlingame, CA) through an analog/digital converter board (Labmaster, Scientific Solutions Inc., Cleveland, OH), using an IBM PC/AT computer. The same software and hardware were used to record the membrane currents at a sampling rate of 25 kHz with 12 bit (12.5 pA) resolution, following analog filtering by a homemade two-pole, low-pass Butterworth filter with a cutoff frequency of 5 kHz. The nodal current was calculated from the feedback amplifier output potential by arbitrarily assuming an internodal resistance of 10 MΩ. The magnitude of the peak inward current at the start of an experiment, using this scaling factor, was typically 10–40 nA for a voltage step from −80 to 0 mV.

Solutions

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 (~ 5 mM). This solution blocked >95% of the "delayed rectifier" K^+ currents. The extracellular solution ("Ringer") 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 Ringer's solution to further inhibit K^+ currents, with no apparent effects on anesthetic block of sodium channels.

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. Concentrated stock solutions in EtOH were stored at −15°C in small aliquots. Individual aliquots were thawed as needed and stored at 4°C for no more than 7 d before use. Drugs were diluted into Ringer as needed; final EtOH concentrations were <1% (vol/vol).

Protocols

Sodium channel function was assessed by monitoring the peak inward sodium current (I_{Na}) during a depolarizing step ("pulse") from the holding potential to either 0 mV or +80 mV. Except where noted, anesthetics were applied at concentrations previously determined to produce 50% tonic inhibition of peak Na current (at a holding potential of −80 mV): this was 200 μM for lidocaine, 25 μM for *d*-bupivacaine. The magnitude of peak I_{Na} after exposure to anesthetic (observed with infrequent stimuli) was taken as a baseline measure of Na channel availability; the binding of anesthetic to the receptor involved in phasic block was assumed nil under these conditions. Any decrease in peak I_{Na} with more frequent stimuli was assumed to represent binding of drug to a fraction of channels equal to the fractional decrease in I_{Na}.

Some binding of LA must occur early in the depolarization, before the Na current has reached a maximum; thus, there is some error involved in the use of peak I_{Na} as the fiduciary point for measure of the availability of functional Na channels *before* a given pulse. I estimated the magnitude of this error by choosing an earlier fiduciary point (midway between the start of the pulse and the time to peak I_{Na}, where less binding has occurred) and comparing the calculated pulse-to-pulse decreases in I_{Na} at this earlier point to those measured from peak I_{Na}. For bupivacaine, the difference was too small to quantify. For lidocaine, under the conditions used in this study, the calculated decrease in I_{Na} from the first to last pulse in a train was as much as 10% greater when the earlier fiduciary point was used, reflecting the rapidity with which lidocaine binds upon depolarization (see Courtney et al., 1978); this finding also suggests that measurements of so-called "tonic" or "resting" block based on decreases in peak I_{Na} may be contaminated by significant binding of drug during activation. No attempts were made to correct for this measurement error in the present study.

The kinetics and voltage-dependence of binding was assessed through three experimental manipulations: changing the duration of depolarizing "test" pulses, changing the amplitude of test pulses, or changing the interpulse (holding) potential. Model parameters were estimated by

fitting of a recursion equation (Appendix, Eq. 9) to peak I_{Na} data observed at three or more pulse rates. The kinetic model is more fully described in the Appendix.

Parameter estimation algorithm

A nonlinear least-squares computer algorithm ("Patternsearch"; Colquhoun, 1971) was used to estimate the model parameters. In brief, initial (arbitrary) parameter estimates were used to generate values of fractional block on a pulse-by-pulse basis, using the recurrence equation 9 of the Appendix for a given interstimulus interval (ISI). These values were then compared to those measured experimentally (as the pulse-to-pulse fractional decrease in peak I_{Na} during a pulse train), and the squared differences were summed. The procedure was repeated for at least two ISIs (usually three or four), the squared differences from all trials summed together. The Patternsearch algorithm was then applied to search for parameter estimates minimizing the squared difference between the model values for block and the observed values. All data points were thus equally weighted by the least-squares procedure. This procedure is similar to a graphical parameter estimation scheme developed previously (Starmer and Grant, 1985), but in practice appeared more robust in fitting the parameters to noisy data.

RESULTS

Recovery kinetics are independent of pulse duration and magnitude

Most models of LA block of Na channels implicitly or explicitly assume that the interpulse rate of recovery of channels from bound states, populated by depolarizing pulses, to the resting, drug-free state is independent of pulse duration and magnitude. If, however, multiple bound states exist without a common rate-limiting pathway for recovery, one would expect longer or larger depolarizations to favor population of bound states with slower recovery kinetics than those states populated by shorter or smaller depolarizations (see Fig. 1). This assumption was tested experimentally by evaluating the effect of pulse potential and duration on the kinetics of recovery between pulses in the presence of either lidocaine or *d*-bupivacaine. Data from one such experiment with 25 μM *d*-bupivacaine are shown in Fig. 2. Phasic block was observed at three rates of stimulation (10 Hz, shown; 4 and 1 Hz, not shown), with a depolarizing pulse potential $E_d = +80$ mV and duration $t_d = 16$ ms. Model parameters were fit to the peak I_{Na} data using the least-squares estimation algorithm described in Methods. The same pulse protocol was then repeated with $E_d = 0$ mV for each stimulation frequency (Fig. 2 *A*). A single recovery rate, $\lambda_r = 0.061\ s^{-1}$, provides a satisfactory fit to the observed decline in peak I_{Na} for both sets of data (continuous curves in Fig. 2 *A*). Similarly, data obtained using pulse durations of 1 ms instead of 16 ms were well fit by the same value of λ_r (Fig. 2 *B*). Experiments using 200 μM lidocaine produced analogous results (data not shown).

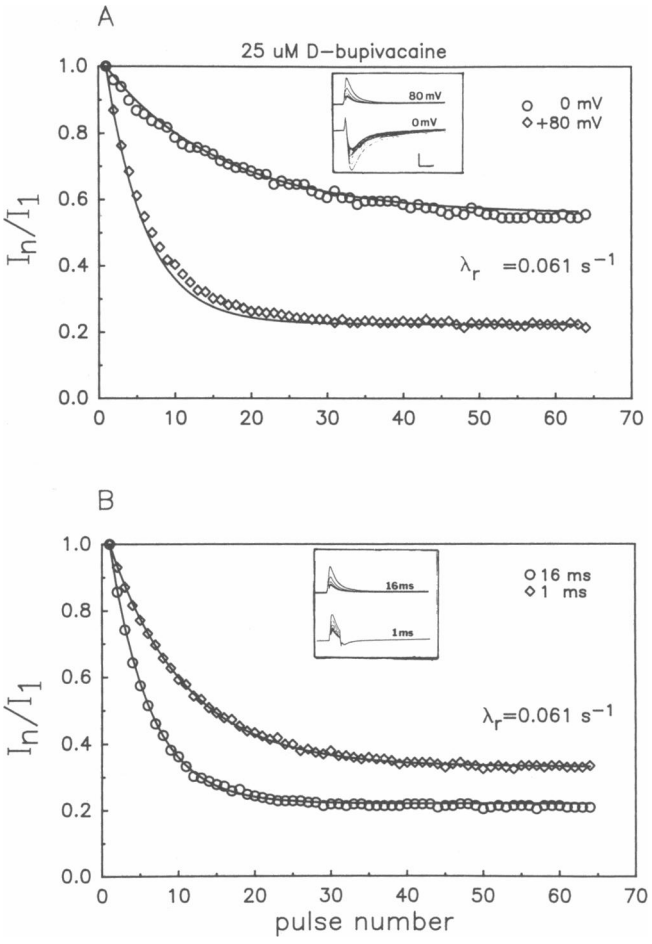

FIGURE 2 The rate of recovery from phasic block by *d*-bupivacaine is independent of pulse potential and duration. (*A*) After several minutes of rest at $E_h = -80$ mV, phasic block was induced by a series of 16-ms pulses to 0 mV (*circles*) or +80 mV (*diamonds*), both applied at 10 Hz. Raw current traces are shown for the first and every fifth subsequent pulse (*inset*). The decreases in peak current were fit by the Courtney model (continuous curves), with a common recovery rate $\lambda_r = 0.061\ s^{-1}$, and pulse-associated parameters $a \cdot k = 0.026$ pulse^{-1}, $a^* \cdot l = 0.026$ pulse^{-1} (0 mV); and $a \cdot k = 0.137$ pulse^{-1}, $a^* \cdot l = 0.032$ pulse^{-1} (+80 mV). Elevated internal Na^+ (35 mM) was used to produce larger outward currents. Parameter estimates derived from data shown together with data (not shown) from separate trials at 4 and 1 Hz. (*B*) Longer pulses potentiate block without affecting the recovery rate. The sodium current response to a train of pulses to +80 mV, with an interstimulus interval of 100 ms, is shown for pulse durations of 16 ms (*circles*) or 1 ms (*diamonds*). Recovery rate $\lambda_r = 0.061\ s^{-1}$. Pulse parameters $a \cdot k = 0.137$ pulse^{-1}, $a^* \cdot l = 0.032$ pulse^{-1} (16-ms pulses); and $a \cdot k = 0.065$ pulse^{-1}, $a^* \cdot l = 0.026$ pulse^{-1} (1-ms pulses). 25 μM *d*-bupivacaine, Fiber 87818A. Scale bars, 5 nA, 1 ms.

These results are consistent with the assumption that the variation in the rate of development and extent of phasic block observed at various pulse potentials and durations is dominated by differences in the rate or extent of net binding during a pulse and not by differences in the rate at

which channels recover between pulses. Thus, it appears that recovery from phasic block at the holding potential can be well characterized by a single rate constant, independent of the protocol used to produce the block.

Concentration dependence of binding parameters

A second testable prediction of the proposed kinetic model is that the on-rates for LA binding should be proportional to, and the off-rates independent of, drug concentration. Therefore, the weighted sum of on-rates, $a \cdot k$, should also be proportional to, and the weighted sum of off-rates, $a^* \cdot l$, independent of concentration. This prediction was examined in experiments with lidocaine and bupivacaine. For lidocaine, phasic block data from separate experiments using either 50 or 200 μM extracellular drug were analyzed, parameters were fit as described above, and the parameter values pooled (Table 1). For bupivacaine, each of three nodes was exposed to successively higher concentrations of drug and phasic block data recorded at each concentration. Both drugs yielded a forward binding rate ($a \cdot k$) that clearly increased as drug concentration was increased; the small sample size and large interexperiment variation, however, prevents quantitative comparison. The reverse binding rate ($a^* \cdot l$), in contrast, was independent of concentration, as was the recovery rate between pulses. These results provide tentative support of a model in which there is a simple 1:1 association of drug and receptor to form a nonconducting complex. Furthermore, the results support a physical interpretation of the highly lumped model rate parameters, $a \cdot k$ and $a^* \cdot l$, as representing association and dissociation of drug, respectively.

TABLE 1 Concentration dependence of kinetic parameters

Concentration	$a \cdot k$	$a^* \cdot l$	λ_r	n
μm				
Lidocaine				
50	0.040 ± 0.002*	0.050 ± 0.005	0.594 ± 0.039	3
200	0.098 ± 0.051	0.058 ± 0.008	0.680 ± 0.123	6
d-Bupivacaine				*Experiment*
25	0.026	0.039	0.033	87730
50	0.055	0.040	0.033	
10	0.025	0.051	0.087	88404‡
25	0.068	0.062	0.079	
25	0.018	0.042	0.400	
50	0.038	0.042	0.410	87N04‡
100	0.076	0.044	0.400	

Holding potential, −80 mV; pulse potential, 0 mV; pulse duration, 16 ms. *Mean ± SEM. ‡Extracellular pH was set at 8.5 in this experiment.

Pulse-duration dependence of binding parameters

As derived in the Appendix, the rate parameters $a \cdot k$ and $a^* \cdot l$ represent the integrated (nondimensional) availability of "bindable" and "unbindable" conformations, a and a^*, multiplied by the respective mean binding and unbinding rates for those conformations, k and l. a^* differs from a in the model because the gating of LA-bound channels is assumed to differ from that of unbound channels (Courtney, 1975), an assumption supported by data of macroscopic (Hille, 1977*b*; Meeder and Ulbricht, 1987) and gating currents (Cahalan and Almers, 1979*a*; Neumcke et al., 1981; Bekkers et al., 1984; Dubois and Schneider, 1985). Although one cannot obtain unique estimates of the availability integrals and binding rates for the individual channel states from macroscopic phasic blocking data, it is possible to follow the time course of the binding reactions by estimating the values of $a \cdot k$ and $a^* \cdot l$ as a function of increasing pulse duration (t_d), thereby selecting for states that occur early during a depolarization (brief pulses), and then adding contributions to the binding reactions from the later occurring states (longer pulses). This was accomplished by applying the parameter estimation procedure to peak I_{Na} data from pulse trains (1–10 Hz) in which t_d was varied from 1 to 32 ms in separate trials. Estimates of $a \cdot k$, $a^* \cdot l$, and λ_r in the presence of 200 μM lidocaine were then plotted against pulse duration (Fig. 3 *B*). Experiments with bupivacaine (data not shown) yielded qualitatively similar results. As in Fig. 2, there was little dependence of the recovery rate constant, λ_r, on t_d. The on-rate parameter, $a \cdot k$, increased monotonically with longer depolarizations, suggesting that bindable conformations continue to be available even after the Na conductance (i.e., the fraction of open channels) has become negligible. In contrast, the pulse-associated unblocking parameter, $a^* \cdot l$, remained relatively constant over the observed range of pulse duration.

Estimation of open channel and inactivated channel binding rates

The experiments and analysis described in the preceding section serve to reveal the time-course of anesthetic binding and unbinding during a depolarization, without regard to individual channel states. Although the rapidly varying mixture of channel states during a depolarization complicates the estimation of *state-specific* rate constants, I will now describe a procedure to obtain these rate constants from the data of Fig. 3, using the following simplifying approximations:

(*1*) I will assume initially that only two channel conformations, open (O) and inactivated (I), participate

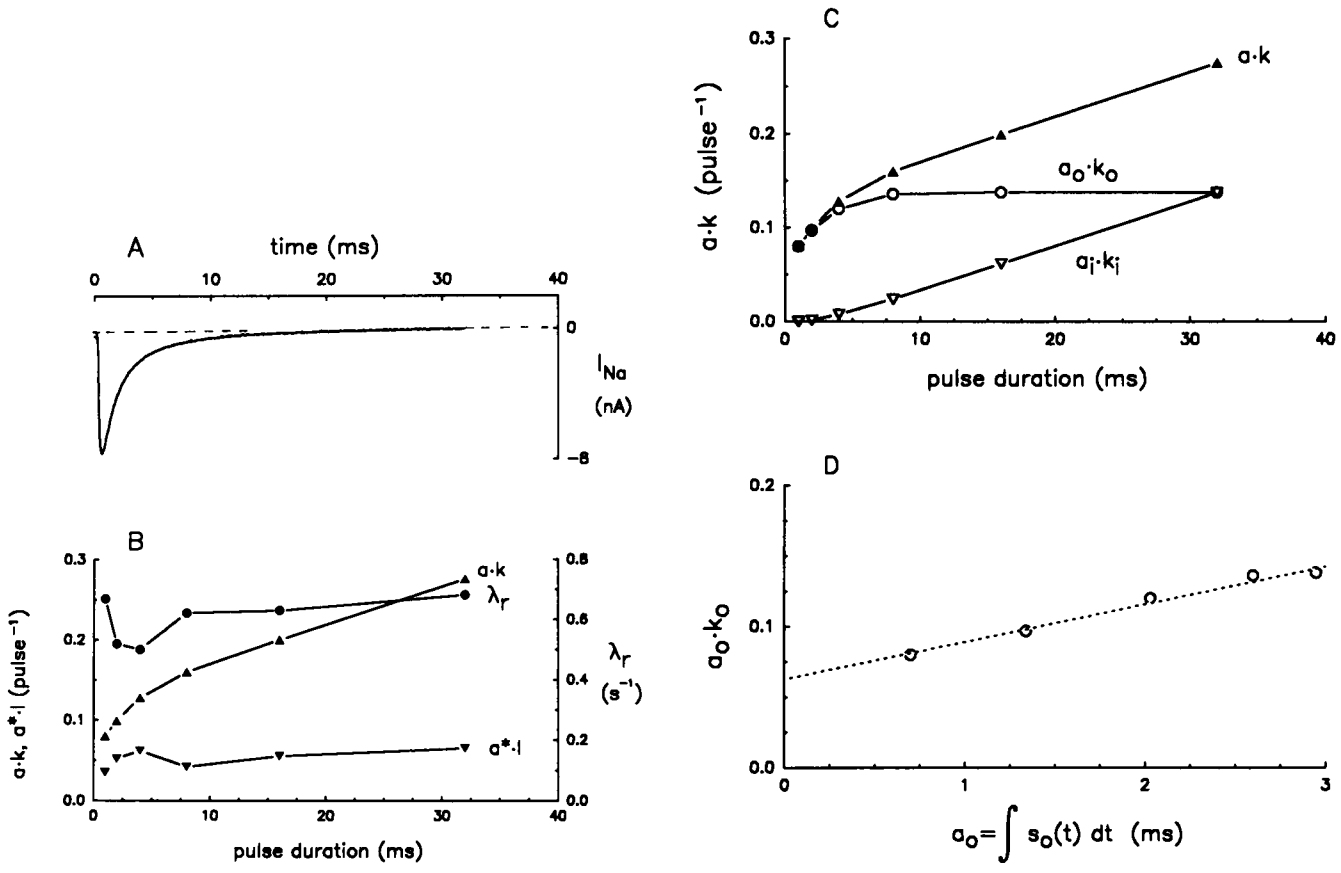

FIGURE 3 Dependence of model parameters on pulse duration. (*A*) Time course of I_{Na} during a single 32-ms-long pulse to 0 mV ($E_h = -80$ mV). (*B*) In the presence of 200 μM lidocaine, phasic block at 10 Hz stimulation rate was elicited for pulse durations of 1, 2, 4, 8, 16, or 32 ms (six separate runs in all). The runs were then repeated at stimulation rates of 4 and 1 Hz. The three runs (10, 4, and 1 Hz) obtained at a single pulse duration were used to generate parameter estimates; these estimates are graphed as a function of pulse duration. The fraction of unbound channels becoming bound with each pulse, $a \cdot k$ (▲), increases monotonically with pulse duration, whereas the fractional unblock per pulse, $a^* \cdot l$ (▼), and the rate of unbinding between pulses, λ_r (●) are relatively independent of pulse duration. The time course of I_{Na} during a single 32-ms pulse is shown in the upper panel. All pulses from −80 to 0 mV. Fiber 87N23. (*C* and *D*) Graphical means of calculating forward binding rates of lidocaine to open and inactivated channels. In *C*, the binding term $a \cdot k$ (from *B*) is separated into terms $a_O \cdot k_O$ and $a_I \cdot k_I$ according to the methods described in the text. k_I is calculated as the slope of $a \cdot k$ curve for $t > 16$ ms, when nearly all channels are inactivated and a_I is linearly related to time. In *D*, the $a_O \cdot k_O$ data from *C* is plotted against a_O, the integral of open channel availability. The slope of the least-squares linear fit through these points, 27.7 s^{-1}, is equal to k_O. The *y*-intercept represents a residual component of binding occurring early in a depolarization and not attributable to open channel binding (see text).

in binding during a depolarizing pulse (this assumption will be reexamined below). This permits simplification of Eq. 5 of the Appendix to include only rate constants from O and I states:

$$a \cdot k = \int_0^t s_O(t) k_O\,dt + \int_0^t s_I(t) k_I\,dt$$
$$= a_O \cdot k_O + a_I \cdot k_I.$$

(*2*) I will assume that the pulse can be divided into three regimes: (*i*) $t < t_{peak}$ (time to peak I_{Na}, during which few channels inactivate [Kniffki et al., 1978] and thus binding is to open channels alone; (*ii*) $t > 16$ ms, during which nearly all channels are inactivated (and thus binding occurs to inactivated channels alone); and (*iii*) $t_{peak} < t < 16$ ms, during which a mixed population of open and inactivated channels are present.

(*3*) I will make the approximation that all channels are in the open state at t_{peak}, and that the fraction of channels in the open state at any other time, t, is simply equal to the ratio of $I_{Na}(t)$ to $I_{Na}(t_{peak})$:

$$s_O(t) = I_{Na}(t)/I_{Na}(t_{peak}).$$

(*4*) I will assume that the decline in I_{Na} after t_{peak} in these nodes is produced primarily by rapid inactivation of open channels (Gonoi and Hille, 1987; Strichartz et al., 1987). Then, given (*3*) above, the fraction of channels in

the inactivated state is simply

$$s_I(t) = 1 - s_o(t), \quad \text{for } t > t_{peak}.$$

Given these approximations, the forward rates of binding to the open and inactivated states can be derived by a stepwise "peeling" process as follows:

(*a*) $t > 16$ ms. The rate of binding (k_I) to the inactivated state I is found by calculating the slope of the least-squares line through the data points $a \cdot k$ for $t > 16$ ms ($a_I \sim 1$). This yields a line with slope $k_I = 4.75\ s^{-1}$ (Fig. 3 *C* and Table 2).

(*b*) $t_{peak} < t < 16$ ms. During this interval the fraction of inactivated channels is changing rapidly, so the rate of binding to inactivated channels is weighted by their availability:

$$a_I \cdot k_I = k_I \int_{t_{peak}}^{t} s_I(t)\, dt.$$

From the previously determined value for k_I and the integral of $s_I(t)$, $a_I \cdot k_I$ can be calculated at $t = 1$, 2, 4, and 8 ms (Table 2).

(*c*) Finally, subtracting $a_I \cdot k_I$ from $a \cdot k$ yields a residual which, from assumption (*1*), is $a_O \cdot k_O$, the open channel contribution to binding (Fig. 3 *C*). To obtain the open channel binding rate k_O, this residual is plotted against a_O, the open channel integral, using assumption (*3*). The relationship is well approximated by a line with slope $k_O = 27.7\ s^{-1}$ (Fig. 3 *D*). Interestingly, the *y*-intercept of this line, 0.061 pulse^{-1}, represents binding unaccounted for by interaction with open or inactivated channels, and is a considerable fraction of the total binding that occurs during a long pulse (see Table 2). A similar analysis of data from one experiment with *d*-bupivacaine yielded rate constants $k_O = 6.2\ s^{-1}$ and $k_I = 1.1\ s^{-1}$, with a residual binding term of 0.009 pulse^{-1}. One plausible explanation is that this residual fraction represents nonconducting but high-affinity "pre . . . open" states through which the sodium channel passes during activation (Horn and Vandenberg, 1984; Chernoff and Strichartz, 1989). A similar hypothesis has been advanced by Gilliam et al. (1989) to account for rapid block of I_{Na} at subthreshold potentials in rabbit atrial myocytes.

TABLE 2 Resolution of forward rate constants for lidocaine binding to open and inactivated channels

t	$a \cdot k$	−	$a_I \cdot k_I$*	=	$a_o \cdot k_o$	a_o‡
ms						*ms*
1	0.080		0.0001		0.080	0.70
2	0.099		0.0016		0.097	1.84
4	0.128		0.0078		0.120	2.03
8	0.160		0.024		0.136	2.60
16	0.200		0.062		0.138	2.95
32	0.276		0.138		0.138	2.95

Data from Fig. 3, this paper. *$k_I = 4.75\ s^{-1}$ determined by slope of line joining $a \cdot k$ data at $t = 16$ and 32 ms ($s_I[t] = 1$ for $t > 16$ ms). ‡$a_o = \int_0^t s_o(t)\, dt$. s_o assumed equal to unity when $I_{Na}(t)$ is maximal.

The bimolecular forward rate constants for lidocaine and bupivacaine binding to open and inactivated channels can be estimated by dividing the derived rates, k_O and k_I, by the concentration (more properly, the activity) of drug in the "vicinity" of the LA binding site. This activity may not be that in the solution bathing the node, because the local anesthetic binding site is thought to be inaccessible directly from the outer surface of the membrane (Hille, 1977*b*). But if the binding site is directly accessible from the axoplasm, (Narahashi et al., 1970; Frazier et al., 1970; Strichartz, 1973; Cahalan, 1978) and the LA has equilibrated across the membrane (see Dettbarn, 1962; Hille, 1977*a*) then the extracellular activity may be used, as has been assumed in other studies (Clarkson and Hondeghem, 1985; Starmer, 1987). For lidocaine, without regard for the degree of ionization, this yielded forward rate constants for open and inactivated channels: $k_O = 1.4 \cdot 10^5\ M^{-1}s^{-1}$ and $k_I = 2.4 \cdot 10^4\ M^{-1}s^{-1}$; and for bupivacaine, $k_O = 2.5 \cdot 10^5\ M^{-1}s^{-1}$, $k_I = 4.4 \cdot 10^4\ M^{-1}s^{-1}$.

The off-rate constants, l_O and l_I, cannot be determined reliably from the data of Fig. 3; this is due in part to the nonmonotonic nature of the $a^* \cdot l$ curve and in part because the kinetics of gating of anesthetic-bound channels (and thus a^*) are not known and so cannot be factored out. Therefore, the apparent equilibrium dissociation constant, $K_a = l/k$, for each channel state is not accessible by this method.

Lidocaine block of Na currents after chloramine-T treatment

An independent means of estimating the binding kinetics of lidocaine during a depolarizing pulse was achieved through analysis of LA block of Na currents after chloramine-T (CT) treatment to partially remove fast Na inactivation (Wang, 1984). As a consequence of the increased availability of open channels during long depolarizations after CT treatment, it was possible to observe lidocaine binding directly as a time-dependent block of the steady-state ("noninactivating") component of I_{Na} (Wang et al., 1987; see also Cahalan, 1978), and obtain binding rate constants from the equilibrium block obtained and the rate of approach to equilibrium.

Nodes of Ranvier were exposed to 0.5 mM external CT, a maintained Na current developing rapidly over the first 5 min and slowly thereafter. CT was washed out after 5–10 min of exposure, and control recordings of Na current responses to single long depolarizations or repeti-

tive short depolarizations were made. These protocols were then repeated after wash-in of LA.

Fig. 4 *A* shows the current response of a CT-treated node to a single 1-s-long depolarizing pulse, first in the absence of drug, and then after successive applications of 100, 200, 400, and 800 μM lidocaine. The potency of lidocaine for tonic block, as assessed by inhibition of peak I_{Na}, was similar to that seen in unmodified channels. The noninactivating current was inhibited nearly completely even at the lowest lidocaine concentration used, and in a time-dependent manner (Fig. 4 *B*). At 400 and 800 μM concentrations, both a fast and a slow component of current decay were observed; at 100 and 200 μM, only a slow component was observed. By fitting a single exponential to the slow component of decline of I_{Na}, an estimate of the time constant for binding at 0 mV was made for each drug concentration: these were 165 ms (100 μM), 107 ms (200 μM), 66 ms (400 μM), and 51 ms (800 μM). An estimate of equilibrium block at each concentration was made by measuring the remaining I_{Na} (relative to that seen in the absence of lidocaine) at the end of the 1-s pulse. This block was nearly complete even at the lowest lidocaine concentration tested, suggesting that the K_d for the open channel is much less than 100 μM. Estimation of the apparent forward rate constant, k_O, for binding to the open state at 0 mV was achieved by assuming continuous availability of the open state during the pulse: k_O estimated in this way ranged from $2.5 \cdot 10^4$ to $6.1 \cdot 10^4$ $M^{-1}s^{-1}$, two- to fivefold lower than estimated from phasic block in non–CT treated node (but see Discussion). The reverse rate constant, l_O, was estimated to be 1.3–1.7 s^{-1}.

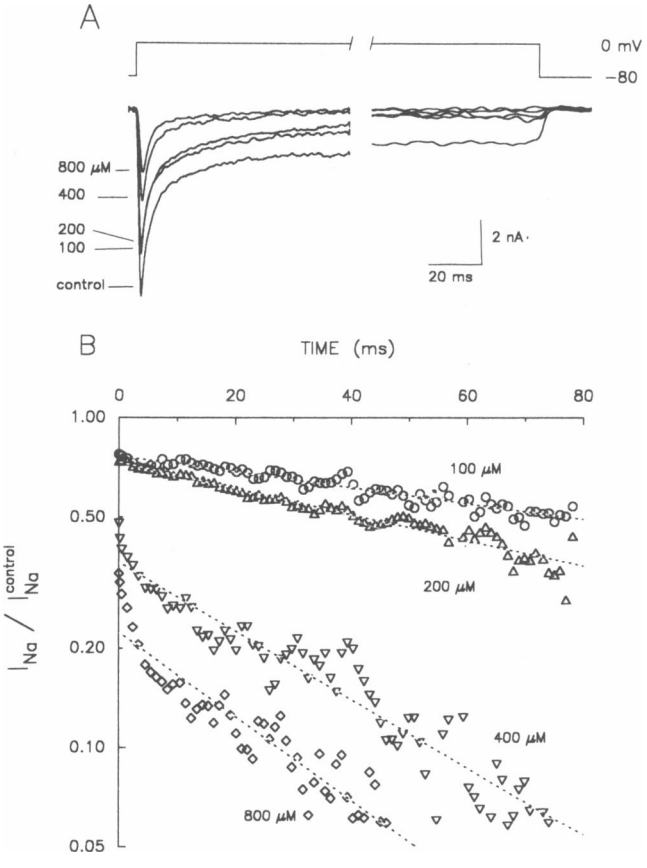

FIGURE 4 The effect of varying concentrations of lidocaine on time-dependent block of Na currents in a CT-treated node. (*A*) The Na current response to an 80-mV, 1-s-long depolarization from the holding potential after CT treatment, in the presence of lidocaine at the following concentrations (in order of decreasing current size): no drug, 100, 200, 400, and 800 μM. Only the first and last 80 ms of each I_{Na} record is shown. (*B*) the declining phase of I_{Na} at each lidocaine concentration are plotted on semilog scales, where the currents have been normalized on a sample-by-sample basis to the current measured in the absence of drug; time zero indicates the time of peak current amplitude. A slow component was fitted by a least-squares linear model to these points (dotted lines), with time constants of 165 ms (100 μM), 107 ms (200 μM), 66 ms (400 μM), and 51 ms (800 μM). Fiber 88322B.

Dependence of binding kinetics on holding potential

The rate of recovery from use-dependent block with a number of anesthetic agents has been reported to depend on the repolarization (holding) potential. For quaternary amine (4°, permanently charged) anesthetics, data from several studies are in good agreement. With the 4° lidocaine analogue QX-314, the rate of recovery from block produced by large depolarizations is accelerated by small depolarizing pulses, consistent with a model in which QX-314 is trapped on its binding site when the Na channel is in the resting, closed conformation but can escape when the channel is open (Strichartz, 1973). In similar experiments in squid giant axon, where the rate of recovery after a train of depolarizing stimuli was measured as a function of holding potential, membrane hyperpolarization was shown to slow markedly (*e*-fold per 14 mV hyperpolarization) the recovery from block with QX-314, with the homologous trimethyl lidocaine derivative, QX-222, and with 9-aminoacridine (a 3° amine drug with $pK_a > 10$, so that it is nearly completely ionized at physiological pH) (Yeh and Tanguy, 1985). In contrast, little voltage dependence of recovery was observed with the tertiary amine LA, lidocaine.

I assessed the voltage dependence of the rate of recovery from phasic block in the node of Ranvier in the following experiments. Phasic block of Na channels was elicited by test depolarizations (to a common potential) from holding potentials (E_h) ranging from −70 to −100 mV, in the presence of a constant extracellular concentration of LA (Fig. 5). For lidocaine (Fig. 5 *A*) and *d*-bupivacaine (Fig. 5 *B*), less-negative holding potentials reduced the fraction of sodium channels available to

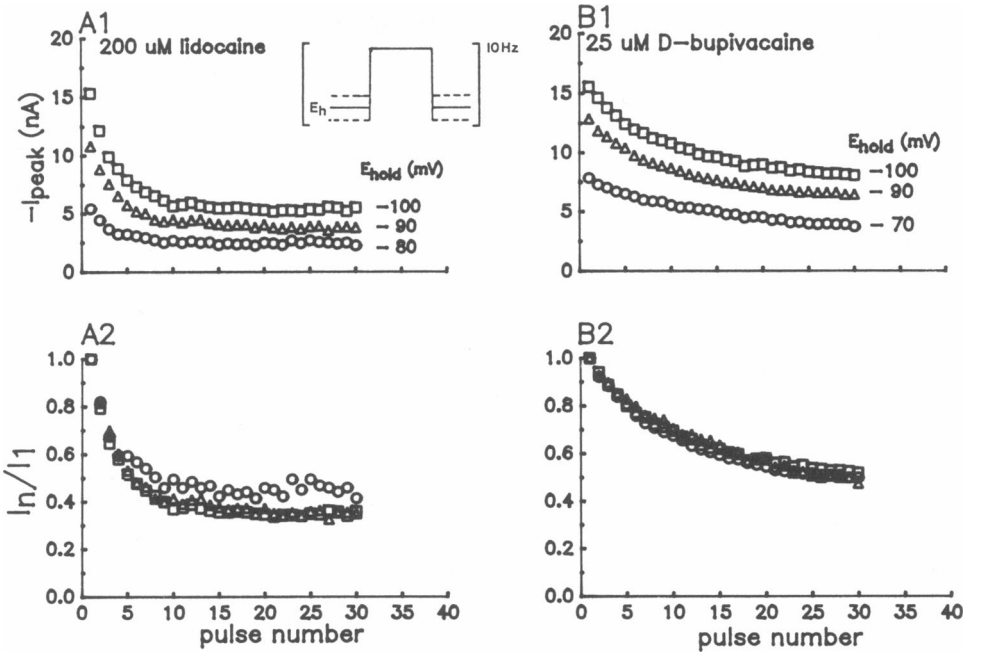

FIGURE 5 Holding potential dependence of phasic block. Trains of depolarizations (16 ms, 0 mV, 10 Hz) were applied at a given holding potential (E_h). The holding potential was changed, 2–3 min allowed for reequilibration, and the pulse train was reapplied. The practical range of holding potentials was limited in the depolarizing direction by increasing inactivation and tonic block, i.e., loss of signal, and in the hyperpolarizing direction by membrane instability. Peak currents are plotted vs. pulse number for each of three holding potentials, in the presence of lidocaine (*A1*) and *d*-bupivacaine (*B1*). (*A2* and *B2*) The same data is replotted, now normalized by the magnitude of the first peak current in the train. With both lidocaine and bupivacaine, there was little change in the time course of phasic block, despite significant changes in tonic block and/or inactivation. This result also held at 4 and 1 Hz stimulation frequencies (data not shown). Experiment identification: lidocaine, 87N23; *d*-bupivacaine, 87N22B.

conduct in response to depolarization, representing the potential dependence of resting inactivation (*top panels*). However, when the data are normalized by the tonically available peak current (Fig. 5, *bottom panels*), it becomes apparent that neither the kinetics nor steady-state amount of phasic block are greatly influenced by E_h. Data obtained at other stimulation rates showed a similar insensitivity of phasic block to holding potential. Application of the Courtney model to these data, along with data obtained at two other stimulus frequencies (not shown), produced estimates of $a \cdot k$, $a^* \cdot l$, and λ_r that varied by <10% over the voltage range investigated. This observation runs counter to the expectations of both modulated receptor models (see Fig. 11 of Bean et al., 1983) and guarded receptor models (see Fig. 5 of Starmer et al., 1984), in which increasing the fraction of bound channels by setting the holding potential less negative decreases the steady-state phasic block. Also, the rate of recovery from phasic block with lidocaine and bupivacaine is not strongly dependent on holding potential in this preparation: this may reflect a rate-limiting step involving deprotonation of drug (presumed voltage independent; see the following paper, Chernoff and Strichartz, 1990).

Long-pulse experiments: voltage dependence of recovery from block induced by a single long depolarization

The results described in the preceding paragraphs indicate that the kinetics of recovery from phasic block in the presence of lidocaine are not strongly affected by the holding potential. This conclusion, while consistent with data from squid giant axon (Yeh and Tanguy, 1985), is at odds with results of similar experiments in heart (e.g., Bean et al., 1983). One possible source for these differences lies in the exact methodology used to induce block of sodium channels: Bean et al. used a single, very long (2 s) depolarization to induce block of sodium channels, whereas Yeh and Tanguy (1985), in common with the present study, used bursts of brief (1–50 ms) pulses. It is not clear that the blocks produced by these different protocols are equivalent. For example, there may be a specific interaction of lidocaine with a slow inactivated state of the sodium channel (Khodorov et at., 1976); such an interaction would be favored by the long depolarizations used by Bean et al., but would be less pronounced with the short

pulses used here (see Fig. 3). To investigate this point, I measured the recovery of peak I_{Na} following single long pulses as a function of holding potential (Fig. 6), using essentially the same protocol employed by Bean et al. In control records performed in the absence of drug, a small amount of slow inactivation was observed, seen as an exponential component of recovery with time constant $\tau = 590$ ms at -90 mV. In the presence of 50 μM lidocaine, the slow component became much larger and the time constant for recovery became longer ($\tau = 1.5$ s), similar to both the time constant observed by Bean et al. at -105 mV ($\tau = 1.7$ s), and the recovery time constants (λ_r) from brief pulses observed in the present study (see Fig. 2). However, unlike the observations made by Bean et al., but consistent with the data shown in Fig. 5 of this study, little or no change in the rate of recovery from block was seen upon hyperpolarization. I conclude that (*a*) recovery from block produced with long depolarizations is kinetically indistinguishable from block produced with brief depolarizations, and (*b*) Na channels in the two preparations interact with lidocaine in a different manner or with different rate-limiting kinetics (see Discussion).

DISCUSSION

The primary conclusion drawn from the work presented in this study is that an empirical piecewise–linear kinetic description of LA binding to Na channels, first described by Courtney (1975), can be used to probe the detailed time course of phasic block, without the need to invoke a specific model (modulated receptor or guarded receptor). Courtney's model is re-derived and extended to the general case of multiple bound states (see Appendix). Analytic tools are described that provide "best" fits (in a least-squares sense) of the model parameters to the observed phasic reductions in peak I_{Na}. The model appears to be adequate for describing recovery from lidocaine and bupivacaine block under a wide range of stimulus protocols; it also is in agreement with the observed concentration dependence of the net "on" and "off" rates of LA during a depolarization. Given several simplifying assumptions regarding the time-dependent availability of channel states during a depolarizing pulse, apparent binding rates to open and inactivated states have been obtained. In the following sections, some of the specific results obtained through the use of these protocols and parameter estimation procedures will be discussed.

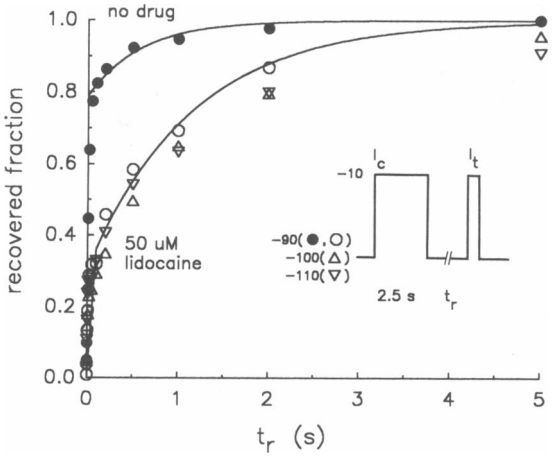

FIGURE 6 Voltage dependence of recovery from block induced by long depolarizations. Solid curves are $1 - A \exp(-t/\tau)$, with $\tau = 590$ ms, $A = 0.21$ at -90 mV in the absence of drug; $\tau = 1.5$ s, $A = 0.70$ in the presence of 50 μM lidocaine (fit to data at $E_h = -90$ mV). Hyperpolarizing to -100 and 110 mV had minimal effect on recovery. Fiber 88519B.

Recovery rate constant (λ_r)

As the Courtney model predicts, the recovery rate between pulses, λ_r, appears independent of the size and duration of the pulses (Figs. 2 and 6); furthermore, λ_r is nearly independent of holding potential (Fig. 5). How do these observations fit with the general kinetic scheme of Fig. 1? Several hypotheses may be considered: (*a*) only a single state binds LA to a significant extent; (*b*) the dissociation rates of LA from all states are similar; (*c*) all channels bound during a depolarization move rapidly to a single common state, e.g., *IL*; (*d*) the dissociation step occurs via a common rate-limiting pathway for all bound states, e.g., $IL \rightarrow I + L$ or $RL \rightarrow R + L$.

The first hypothesis is unlikely given the complex time course of block with lidocaine (Fig. 9 of Bean et al., 1983; Fig. 3 of this paper), which fails to parallel either the time course of activation or inactivation of channels. Hypothesis *b* is possible; however, it is incompatible with the modulated receptor hypothesis, in which inactivated states bind LA more tightly than resting states (as reflected by a decrease in the off-rate). Hypothesis *c* also cannot be ruled out by the data presented: there are at present no available estimates of the rate of transition of *RL* and *OL* to *IL*. The pulse durations used in this study may not have been sufficiently brief to "capture" large numbers of channels in *OL* at the moment of repolarization, for example, so that for most channels, recovery started from the *IL* state. Finally, hypothesis *d* is consistent with the results reported here, those previously reported by Wang et al. (1987) for squid giant axon, and with the pH dependence of recovery from block discussed in the next paper (Chernoff and Strichartz, 1990).

Which transition is rate limiting for recovery?

The problem of determining the sequence of states that depolarized, anesthetic-bound channels traverse before arriving at the unbound resting state has been discussed in detail in a review by Hondeghem and Katzung (1984). Assuming that most LA-bound channels equilibrate in the IL state at the end of a long pulse, these authors identify two likely sequential pathways for recovery at rest:

$$(i)\ IL \rightarrow RL \rightarrow R + L; \quad \text{or}$$

$$(ii)\ IL \rightarrow I + L \rightarrow R + L.$$

Three of the transitions ($IL \rightarrow RL$, $RL \rightarrow R + L$, or $IL \rightarrow I + L$) in the above sequences may in principle become a rate-limiting step in recovery from phasic block; the fourth transition, $I \rightarrow R$, is known to be rapid at normal resting potentials. The experiments described in the present study may help to resolve the relative rates of transition between states for lidocaine and bupivacaine.

$IL \rightarrow RL$

The voltage dependence of the rate of recovery from block has been used, within the framework of a modulated receptor hypothesis, to distinguish between rate-limiting pathways (see Hondeghem and Katzung, 1984). If the voltage dependence of inactivation for LA-bound channels is simply a voltage-shifted version of that for normal channels, as the modulated receptor requires, then the rate of translocation $IL \rightarrow RL$ will be strongly voltage-dependent around the resting potential (analogous to the strong voltage dependence of $I \rightarrow R$). The lack of voltage dependence of recovery with lidocaine and bupivacaine observed in the present study (Figs. 5 and 6) is inconsistent with a rate-limiting $IL \rightarrow RL$ transition, and indeed suggests that, for these drugs, dissociation is also relatively voltage independent over the 30-mV range of potentials tested. These results are similar to those reported in squid giant axon for lidocaine by Yeh and Tanguy (1985); however, studies in cardiac muscle (Chen et al., 1975; Hondeghem and Katzung, 1977; Bean et al., 1983) instead found significant acceleration of recovery from lidocaine block with membrane hyperpolarization. These contradictory findings remain unresolved; however, rather than reflecting basic differences in the mode of action of lidocaine in different tissues, they may instead simply reflect different rate-limiting steps in recovery of drug-bound channels to the resting state.

$RL \rightarrow R + L$

It has been argued elsewhere (Hondeghem and Katzung, 1984) that pathway (i) becomes an important route for recovery only when the membrane is sufficiently hyperpolarized to overcome the (putative) shift in the voltage dependence of inactivation induced by anesthetic binding. This condition was not satisfied in the present study; because 10–20% of unbound channels were inactivated at the usual holding potential (−80 mV), even a modest hyperpolarizing shift in the voltage dependence of inactivation should have caused most drug-bound channels to equilibrate in the IL state at rest. Therefore, if $RL \rightarrow R + L$ is indeed the rate-limiting step in recovery then either (a) there is little voltage-shift of inactivation in drug-bound channels (inconsistent with the modulated receptor hypothesis but consistent with a guarded receptor); or (b) the shift is caused by an increase in the $RL \rightarrow IL$ transition rate rather than by a decrease in the reverse transition rate (so that $IL \rightarrow RL$ does not become rate limiting for unblock of channels).

$IL \rightarrow L + L$

If $IL \rightarrow I + L$ is rate limiting for recovery and is independent of potential, then little voltage dependence of recovery is predicted, even in the presence of a large voltage-shift in inactivation of drug-bound channels (see Hondeghem and Katzung, 1984). The main objection to a rate-limiting role for this transition raised in this study is the presence of a significant amount of use-dependent unbinding of drug ($a^* \cdot l$); if few channels are in the RL state just before depolarization (as expected if most channels recover from block via $IL \rightarrow I + L$), little use-dependent unblock should occur.

Pre-open channel binding

In Results, the time course of binding of local anesthetic during a depolarizing pulse was deconvolved, using a very simple binding model, into the contributions from open, inactivated, and putative "pre-open" states. The rate of binding of LA to the pre-open state(s) appeared very high: for lidocaine, the amount of block accumulating prior to the appearance of measurable Na current represented ~25% of the total block produced by a long depolarization. Although I have not pursued this putative pre-open state–LA interaction further in this study, a similar conclusion has been reached elsewhere: Gilliam et al. (1989) found significant block of I_{Na} in rabbit atrial myocytes by lidocaine at subthreshold potentials; Chernoff and Strichartz (1989) demonstrated in toad nerve that, in the presence of the lidocaine homolog glycinexylidide, repetitive pulses only 0.2 ms in duration (too short to

open or inactivate a significant fraction of channels) produced nearly as much block of I_{Na} as repetitive 32-ms pulses. Further quantitative work along these lines promises to enrich our understanding of the state dependence of LA block of Na channels.

Pulse-associated rate parameters ($a \cdot k$ and $a^* \cdot l$)

Much of the preceding analysis presumes that drug-bound channels undergo conformational changes (state transitions) during depolarization that parallel those of unmodified channels. Although no direct measurements of drug-bound state transition rates were possible in this study, some inferences are possible from the pulse duration dependence of the pulse-associated blocking and unblocking parameters, $a \cdot k$ and $a^* \cdot l$. The dependence of these separate parameters on the duration of pulses is dissimilar: $a \cdot k$ increases continuously during a pulse, while $a^* \cdot l$ is relatively independent of pulse duration beyond 10 ms (Fig. 3). Within the context of the model used to generate these parameter estimates, these findings may be explained by hypothesizing that LA-bound channels have $a^* \neq a$, i.e., an altered time- or voltage-dependence of gating (as proposed originally by Courtney, 1975). Channels appear to bind LA continuously during a prolonged depolarization, but most unbinding from channels seems to occur within the first few milliseconds. Based on these findings, I tentatively conclude that drug-bound channels (*a*) pass through the conformational equivalent of the open state where drug escape is rapid, (*b*) inactivate more rapidly than unbound channels (otherwise $a^* \cdot l_O$ would parallel $a \cdot k_O$), and (*c*) allow only a slow rate of escape of drug from the inactivated state.

This interpretation, that drug-bound channels can experience rapid voltage-dependent state transitions, must be reconciled with evidence of gating charge immobilization by quaternary LAs (Cahalan and Almers, 1979*a*, *b*; Bekkers et al., 1984) and by neutral benzocaine (Neumcke et al., 1981; Schneider and Dubois, 1986). That is, if gating charge movement represents the transition of the channel between nonconducting and conducting conformations, then abolition of gating charge movement by anesthetic binding should also abolish the associated conformational transitions. In this context, it is notable that of the LAs and LA-like drugs which have been studied using gating currents, only pancuronium (Armstrong and Yeh, 1978) and *N*-methylstrychnine (Cahalan and Almers, 1979*b*) appear to block gating charge movement completely; quaternary derivatives of lidocaine and the neutral LA benzocaine have been shown to block selectively one of two components of the off-gating current in squid (Bekkers et al., 1984). Many LA agents may therefore allow voltage-dependent conformational transitions of drug-bound channels, albeit with altered voltage dependence and kinetics.

Estimates of binding rates to open and inactivated channels in normal and chloramine-T treated node

In this study, several idealizing assumptions have been used to estimate state-specific binding rate constants from $a \cdot k$, the lumped forward binding parameter described in the model. The open channel binding rates for lidocaine thus obtained (see Results) are surprisingly close to those obtained by others (Schwarz et al., 1977; Starmer, 1987), despite the many differences in techniques, models, and methods of analysis between studies. For example, Schwarz et al. (1977) reported $k_O = 2.5 \cdot 10^5\ M^{-1}s^{-1}$ for lidocaine in frog skeletal muscle; Starmer (1987) reported $k_O = 1.73 \cdot 10^5\ M^{-1}s^{-1}$ for lidocaine, $k_O = 5.65 \cdot 10^5\ M^{-1}s^{-1}$ for bupivacaine, under the assumption that all binding is to open channels. The estimated rate constants for binding of lidocaine and *d*-bupivacaine were within a factor of two, and the ratio of open to inactivated channel rate constants was ~6:1 for both drugs. The significance of the latter two findings is unknown.

The study of LA block of CT-modified Na currents yielded a forward rate constant for binding of lidocaine to open sodium channels (observed directly as a time-dependent decline of steady-state I_{Na}, I_{Na}^{ss}) that may at first glance appear to be free of many of the assumptions and idealizations involved in similar estimates made from observation of phasic block. However, without a microscopic understanding of the behavior of CT-modified channels, this view is unwarranted. The binding rate constant was calculated assuming that the inhibition of I_{Na}^{ss} was produced solely by open channel binding. However, studies of chloramine-T modified currents by others (Nagy, 1987) support the idea that CT does not prevent inactivation but instead allows inactivated channels to reopen. In the context of the data presented in Fig. 4, if we assume that I_{Na}^{ss} represents channels that are in equilibrium between *O* and *I* states (spending about one-fifth of their time in *O*), then the estimated binding rate constant, $2.5\text{–}6.1 \cdot 10^4\ M^{-1}s^{-1}$, must be a weighted sum of the binding rates to *O* and *I*. This concept is supported by the fact that the open and inactivated channel binding rate constants derived from analysis of phasic block (e.g., Fig. 3) bracket the estimate made from block of CT-modified currents: if the open and inactivated channel rate constants for lidocaine derived from Fig. 3 are weighted by the ratio of steady-state to peak I_{Na} in Fig. 4 *A*, the calculated weighted binding rate "constant" is $(0.2) \cdot k_O + (0.8) \cdot l_O = 4.7 \cdot 10^4\ M^{-1}s^{-1}$, in good agreement with that estimated from time-dependent block of I_{Na} above.

Tonic block

An explicit assumption of the kinetic model, as formulated in the Appendix, is that no channels are blocked at rest. This assumption is clearly at variance with the 50% resting block produced at the drug concentrations and holding potentials used here. It is remarkable, then, that this model is able to predict the use-dependent decline in peak I_{Na} with parameters that exhibit the expected dependence on concentration (Table 1). The separation of tonic and phasic block in the model may be justified if these two types of block are produced by independent mechanisms (see, e.g., Elliott et al., 1987). However, it should be possible eventually to incorporate equilibrium binding of drug at rest (tonic block) within the Courtney kinetic model; this will, at the expense of additional complexity, enable more accurate global estimates of all binding and state-transition rates (as has been described previously by Hondeghem and Katzung, 1977).

As a separate consideration, the analysis of the duration dependence of block provides a measure of the adulteration of tonic inhibition by the test pulse itself. Thus, for lidocaine I_{Na} is already depressed by an additional 14% by the fractional binding that has occurred at the time of peak current at 0 mV. Shorter test pulse will reduce the adulteration by this binding, but are limited by the smaller signal amplitude and by the inhibition that may develop before any current flows, as described in Results. Any measurement of tonic block, therefore, includes a phasic contribution whose magnitude depends on the drug concentration and the rapidity with which the LA combines with preopen channel states. For LAs that bind very rapidly, such as glycinexylidide (GX; Chernoff and Strichartz, 1989), almost all of the phasic block develops during the brief (200-μs) pulse that is used to assess the tonic level of block. Contamination by phasic inhibition adds another ambiguity to the already arbitrary separation of tonic and phasic block.

Holding potential independence of phasic block

The lack of effect of holding potential on the kinetics of phasic block with lidocaine and *d*-bupivacaine constitutes a novel finding. This result appears to be at odds with the modulated receptor hypothesis. For example, in arguing for a modulated receptor mechanism for lidocaine block of sodium channels in heart, Bean et al. (1983) proposed, on the basis of mass action, that lidocaine produces less block per pulse at depolarized holding potentials because of "tonic block occlusion". That is, at less negative holding potentials, where tonic block is considerable, most channels are already distributed between drug-free inactivated and drug-bound inactivated states; further inactivation by depolarizing pulses can only slightly increase the occupancy of inactivated states, thus little additional block accrues. Bean et al. also suggested that, when repetitive pulse trains are used to produce phasic block, accelerated recovery from block seen at more negative holding potentials between pulses may offset a greater fractional binding per pulse under these conditions. However, the results presented in Fig. 5 of the present paper show that, with lidocaine and *d*-bupivacaine, neither the fractional binding per pulse nor the rate of recovery (λ_r) between pulses is strongly dependent on E_h. This is also evident in Fig. 6, where the time course of recovery from lidocaine block, measured directly, is insensitive to E_h. Therefore, offsetting changes in the rate of development of block during depolarization and the rate of recovery from block between depolarizations cannot be invoked to account for the lack of E_h dependence of phasic block observed here. This result provides additional evidence that there are true, significant differences in the sodium channel–lidocaine interaction in mammalian cardiac preparations and in amphibian node of Ranvier. Caution is therefore warranted when applying results of LA studies in one tissue to determination of mechanism in another.

The lack of dependence of λ_r on holding potential is probably best explained by proposing that a conformational change in the channel (e.g., $IL \rightarrow RL$) is not rate-limiting (since this conformational shift is presumed voltage dependent). Instead, the deprotonation of bound LA or the actual dissociation of LA from its binding site is likely to represent the rate-limiting step in recovery under the conditions explored here, because these steps may indeed be voltage independent. In the following paper (Chernoff and Strichartz, 1990), the rates of local anesthetic deprotonation and dissociation are estimated through an analysis of the pH dependence of λ_r.

APPENDIX: KINETIC MODELING OF PHASIC BLOCK OF SODIUM CHANNELS BY LOCAL ANESTHETIC

Derivation of model parameters

The differential equation describing first-order binding of a drug to a fixed number of sodium channels is

$$db/dt = k[D](1 - b) - l \cdot b, \quad (1)$$

where b is the fraction of drug-bound channels, $[D]$ is the effective drug concentration at the binding site, and k and l are the forward and reverse binding rate constants (possibly voltage dependent; see Strichartz, 1973).

Binding during depolarization

During a depolarization, there may be u different channel conformations ("states"), $s_1, s_2, \ldots, s_u$, the mixture changing dynamically with time.

If we assume that each state, s_i, interacts with drug with unique rate constants, k_i and l_i, forming a drug-bound state s_i^*. The differential rate of binding, db/dt, can then be expressed, to a first approximation, as

$$db/dt = \sum_{i=1}^{u} [\Pr\{s_i, t\}\, k_i\, (1 - b) - \Pr\{s_i^*, t\}\, l_i\, b], \quad (2)$$

where k_i and l_i correspond to $k[D]$ and l, respectively, for the i'th state, and $\Pr\{s_i,t\}$ is the probability that a channel is in state s_i at time t. If we integrate Eq. 2 over the duration, T, of a depolarization (or "pulse"), we obtain the fractional increase in binding during the pulse, Δb:

$$\Delta b = \int_0^T \sum_{i=1}^{u} [\Pr\{s_i, t\}\, k_i\, (1 - b) - \Pr\{s_i^*, t\}\, l_i\, b]\, dt, \quad (3)$$

or, exchanging the order of integration and summation,

$$\Delta b = \sum_{i=1}^{u} \int_0^T [\Pr\{s_i, t\}\, k_i\, (1 - b) - \Pr\{s_i^*, t\}\, l_i\, b]\, dt. \quad (4)$$

Now, if we define two new parameters,

$$a \cdot k = \sum_{i=1}^{u} \int_0^T \Pr\{s_i, t\}\, k_i\, dt, \quad (5)$$

and

$$a^* \cdot l = \sum_{i=1}^{u} \int_0^T \Pr\{s_i^*, t\}\, l_i\, dt, \quad (6)$$

then we have

$$\Delta b = a \cdot k\, (1 - b) - a^* \cdot l\, b, \quad (7)$$

where $a \cdot k$ and $a^* \cdot l$ have units of pulse^{-1}.

This simplified difference equation (Eq.7; note that these expressions are only first-order approximations of the true differential solutions; that is, they are exact only when a channel which becomes bound during a pulse pulse does not unbind drug later in the pulse or vice-versa) was first derived by Courtney et al. (1978) for binding of local anesthetics to a single (open) state, but as shown above, it is generalizable to multistate binding. As an approximate physical interpretation of Eq. 7, I propose that a and a^* represent the mean, integrated availability of states that are able to bind and unbind drug, respectively; k and l represent the mean association and dissociation rates for 1:1 binding of LA to receptor.

Recovery between pulses

The kinetics of recovery from block between pulses are modeled by assuming that a single step is rate limiting for recovery from block. This rate-limiting step might, for example, be the $A^*L \rightarrow R^*L$ transition in Fig. 1 *B* (i.e., a conformational transition) or the $R^*L \rightarrow R$ transition (i.e., actual dissociation). If a single step is indeed rate-limiting, the recovery from block will proceed as a single exponential, with a recovery rate λ_r (units of seconds^{-1}). If a fraction, b_r, of the binding sites are occupied at rest, and a larger fraction, b_x, are occupied at the end of a depolarization, then the relaxation is described by:

$$b = b_r\, (1 - e^{-\lambda_r t}) + b_x\, e^{-\lambda_r t}), \quad (8)$$

where $\lambda_r = k_r + l_r$, and k_r and l_r are the forward and reverse rate constants for binding to the resting state.

Recursion relationship

With a discrete rule (Eq. 7) describing binding during a depolarizing pulse, and a continuous rule (Eq. 8) describing recovery between pulses, it becomes possible to derive a recursion relation describing the fraction of binding sites occupied just before the nth pulse in a constant-frequency pulse train:

$$b_n = b_{n-1}\, (1 - a \cdot k - a^* \cdot l)\, e^{-\lambda_r t_r} - a \cdot k\, e^{-\lambda_r t_r} + b_r\, (1 - e^{-\lambda_r t_r}), \quad (9)$$

where t_r is the interval between pulses. Eq. 9 is a first-order finite difference equation with constant coefficients. After the treatment of Courtney et al. (1978), the general solution to this difference equation is given by

$$b_n = b_{ss} + (b_r - b_{ss})\, e^{-\Lambda \cdot n}, \quad (10)$$

where b_{ss}, the measurable steady-state block produced by a pulse train, is given by

$$b_{ss} = \frac{a \cdot k e^{-\lambda_r t_r} + b_r\, (1 - e^{-\lambda_r t_r})}{1 - e^{-\Lambda}}. \quad (11)$$

and Λ, the measurable rate of development of block (units of pulse^{-1}), is given by

$$\Lambda = \lambda_r\, t_r - \ln\, (1 - a \cdot k - a^* \cdot l). \quad (12)$$

We can further simplify Eqs 9–12 by (*a*) assuming that resting binding b_r is negligible, i.e., $\lambda_r \ll l_r$ (this assumption requires either that there is negligible tonic block, or that tonic and phasic block are due to separate effects of drug); and (*b*) converting $a \cdot k$ and $a^* \cdot l$ to a relaxation rate, λ_d, and equilibrium binding level, $B_d(\infty)$, by the following transformation:

$$B_d\, (\infty) = a \cdot k/(a \cdot k + a^* \cdot l), \quad (13)$$

$$\lambda_d = a \cdot k + a^* \cdot l. \quad (14)$$

Under these conditions, Eqs. 9–12 become

$$b_n = b_{n-1}\, (1 - \lambda_d)\, e^{-\lambda_r t_r} + \lambda_d\, B_d\, (\infty)\, e^{-\lambda_r t_r}, \quad (15)$$

$$b_{ss} = \frac{k_d\, B_d\, (\infty)\, e^{-\lambda_r t_r}}{1 - (1 - \lambda_d)\, e^{-\lambda_r t_r}}, \quad (16)$$

and

$$\Lambda = \lambda_r\, t_r - \ln\, (1 - \lambda_d). \quad (17)$$

The parameter estimation procedure used in this paper fit Eq. 15 to phasic block data by varying the values of $B_d(\infty)$, λ_d, and λ_r in to minimize the sum of squared error between predicted and observed values. The more "fundamental" parameters $a \cdot k$ and $a^* \cdot l$ were then calculated from Eqs. 13 and 14.

Practical considerations in parameter estimation

There are a number of practical considerations involved in obtaining parameter estimates. These are offered here without rigorous proof:

(*a*) *Number of pulsing frequencies required to estimate the binding parameters.* In theory, two rates of stimulation are sufficient to overspecify the system of equations. There are two independent pieces of

information provided by each pulse train, a relaxation rate and a steady-state level of block. Since there are three model parameters, two stimulation rates suffice (if the model is a perfect description of the system and if there is no measurement error) to determine the model parameters. For real data which includes noise sources and measurement error, it is prudent to obtain data at three or more stimulation rates, both to compensate for noisy data and to provide a more stringent test of the model.

(*b*) *Number of pulses per pulse train.* In general, one should apply enough pulses to reach steady-state binding, because this constrains the model parameters more than if steady-state is not achieved. No advantages accrue when using many more pulses than required to reach steady state. On the contrary, the least-squares estimation scheme used here suffers when too many steady-state data points are included, because these have the effect of weighting the steady state more heavily than the transient portion of the data.

(*c*) *Range of frequencies.* If the stimulations frequencies used are all much lower than the recovery relaxation rate, λ_r, little block will accumulate between pulses and the differences in block between runs will be small. Similarly, if the frequencies are all much higher than λ_r, little recovery will occur between pulses and the differences between runs will again be small. A good rule of thumb, then, is to use frequencies both higher and lower than λ_r, because this practice will ensure steady-state levels of binding, b_{ss}, that differ considerably; this in turn places tighter constraints on the values of the model parameters.

(*d*) *Attainment of equilibrium.* If the system is not at equilibrium before a pulse train is applied, the measurements will be skewed, because the size of the peak current produced by the first pulse is used as the standard to assess Na channel availability before the pulse train. In practice, waiting at least three recovery time constants after a pulse train before applying the next appears to be adequate for Na channels to recover to a resting equilibrium, as judged by a return of I_{Na} to its resting size.

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REFERENCES

Armstrong, C. M., and J. Z. Yeh. 1978. Selective block of "off" gating current. *Biophys. J.* 21:41a. (Abstr.)

Bean, B. P., C. J. Cohen, and R. W. Tsien. 1983. Lidocaine block of cardiac sodium channels. *J. Gen. Physiol.* 81:613–642.

Bekkers, J. M., N. G. Greeff, R. D. Keynes, and B. Neumcke. 1984. The effect of local anesthetics on the components of the asymmetry current in the squid giant axon. *J. Physiol.* (Lond.). 352:653–668.

Cahalan, M. D. 1978. Local anesthetic block of sodium channels in normal and pronase-treated squid giant axons. *Biophys. J.* 23:285–311.

Cahalan, M. D., and W. Almers. 1979*a*. Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin. *Biophys. J.* 27:39–56.

Cahalan, M. D., and W. Almers. 1979*b*. Block of sodium conductance and agating current in squid giant axon poisoned with quaternary strychnine. *Biophys. J.* 27:57–74.

Chen, C.-M., L. S. Gettes, and B. G. Katzung. 1975. Effect of lidocaine and quinidine on steady state characteristics and recovery kinetics of (dv/dt) max in guinea pig ventricular myocardium. *Circ. Res.* 37:20–29.

Chernoff, D. M., and G. R. Strichartz. 1989. Binding to and dissociation from closed neuronal sodium channels by protonated local anesthetics during use-dependent block. *In* Molecular and Cellular Mechanisms of Antiarrhythmic Agents. L. M. Hondeghem, editor. Futura Publishing, Mt. Kisco, NY.

Chernoff, D. M., and G. R. Strichartz. 1990. Kinetics of local anesthetic inhibition of neuronal sodium currents: pH- and hydrophobicity dependence. *Biophys. J.* 58:69–81.

Clarkson, C. W., and L. M. Hondeghem. 1985. Mechanism for bupivacaine depression of cardiac conduction: fast block of sodium channels during the action potential with slow recovery from block during diastole. *Anesthesiology.* 62:396–405.

Colquhoun, D. C. 1971. Lectures on Biostatistics. Clarendon Press, Oxford, UK. 257–272.

Courtney, K. R. 1975. Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. *J. Pharmacol. Exp. Ther.* 195:225–236.

Courtney, K. R., J. J. Kendig, and E. N. Cohen. 1978. The rates of interaction of local anesthetics with sodium channels in nerve. *J. Pharmacol. Exp. Ther.* 207:594–604.

Dettbarn, W. D. 1962. The active form of local anesthetics. *Biochem. Biophys. Acta.* 57:73–76.

Dodge, F. A., and B. Frankenhaeuser. 1958. Membrane currents in isolated frog nerve fibre under voltage clamp conditions. *J. Physiol. (Lond.).* 143:76–90.

Dubois, J.-M. and M. F. Schneider. 1985. Kinetics of intramembrane charge movement and conductance activation of batrachotoxin-modified sodium channels in frog node of Ranvier. *J. Gen. Physiol.* 86:381–394.

Elliott, J. R., D. A. Haydon, and B. M. Hendry. 1987. The mechanisms of sodium current inhibition by benzocaine in the squid giant axon. *Pfluegers Arch. Eur. J. Physiol.* 409:596–600.

Fox, J. M. 1976. Ultra-slow inactivation of the ionic currents through the membrane of myelinated nerve. *Biochim. Biophys. Acta.* 426:232–244.

Frazier, D. T., T. Narahashi, and M. Yamada. 1970. The site of action and active form of local anesthetics. II. Experiments with quaternary compounds. *J. Pharmacol. Exp. Ther.* 171:45–51.

Gilliam, F. R., III, C. F. Starmer, and A. O. Grant. 1989. Blockade of rabbit atrial sodium channels by lidocaine: characterization of continuous and frequency-dependent blocking. *Circ. Res.* 65:723–739.

Gonoi, T., and B. Hille. 1987. Gating of Na channels: inactivation modifiers discriminate among models. *J. Gen. Physiol.* 89:253–274.

Hille, B. 1966. Common mode of action of three agents that decrease the transient change in sodium permeability in nerves. *Nature (Lond.).* 210:1220–1222.

Hille, B. 1977*a*. The pH-dependent rate of action of local anesthetics on the node of Ranvier. *J. Gen. Physiol.* 69:475–496.

Hille, B. 1977*b*. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* 69:497–515.

Hondeghem, L. M., and B. G. Katzung. 1977. Time and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochim. Biophys. Acta.* 472:373–398.

Hondeghem, L. M., and B. G. Katzung. 1984. Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium channel-blocking drugs. *Annu. Rev. Pharmacol. Toxicol.* 24:387–423.

Horn, R., and C. A. Vandenberg. 1984. Statistical properties of single sodium channels. *J. Gen. Physiol.* 84:505–534.

Khodorov, B. I. 1981. Sodium inactivation and drug-induced immobilization of the gating charge in nerve membrane. *Prog. Biophys. Mol. Biol.* 37:49.

Khodorov, B. I., L. Shishkova, E. Peganov., and S. Revenko. 1976. Inhibition of sodium currents in frog Ranvier node treated with local anesthetics: role of slow sodium inactivation. *Biochim. Biophys. Acta.* 433:409–435.

Kniffki, K. D., D. Siemen, and W. Vogel. 1978. Delayed development of sodium permeability inactivation in the nodal membrane. *J. Physiol. (Lond.).* 284:92–93.

Meeder, T., and W. Ulbricht. 1987. Action of benzocaine on sodium channels of frog nodes of Ranvier treated with chloramine-T. *Pfluegers Arch. Eur. J. Physiol.* 409:265–273.

Nagy, K. 1987. Subconductance states of single sodium channels modified by chloramine-T and sea anemone toxin in neuroblastoma cells. *Eur. Biophys. J.* 15:129–132.

Narahashi, T., D. T. Frazier, and M. Yamada. 1970. The site of action and active form of local anesthetics. I. Theory and pH experiments with tertiary compounds. *J. Pharmacol. Exp. Ther.* 171:32–44.

Neumcke, B., W. Schwarz, and R. Stampfli. 1981. Block of Na channels in the membrane of myelinated nerve by benzocaine. *Pfluegers Arch. Eur. J. Physiol.* 390:230–236.

Schneider, M. F., and J.-M. Dubois. 1986. Effects of benzocaine on the kinetics of normal and batrachotoxin-modified Na channels in frog node of Ranvier. *Biophys. J.* 50:523–530.

Schwarz, W., P. T. Palade, and B. Hille. 1977. Local anesthetics: effects of pH on use-dependent block of sodium channels in frog. *Biophys. J.* 20:343–368.

Starmer, C. F. 1987. Theoretical characterization of ion channel blockade. *Biophys. J.* 52:405–412.

Starmer, C. F., and K. R. Courtney. 1986. Modeling ion channel blockade at guarded binding sites: application to tertiary drugs. *Am. J. Physiol.* 251:H848–H586.

Starmer, C. F., and A. O. Grant. 1985. Phasic ion channel blockade: a kinetic model and parameter estimation procedure. *Mol. Pharmacol.* 28:348–356.

Starmer, C. F., A. O. Grant, and H. C. Strauss. 1984. Mechanisms of use-dependent block of sodium channels in excitable membranes by local anesthetics. *Biophys. J.* 46:15–27.

Strichartz, G. R. 1973. The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *J. Gen. Physiol.* 62:37–57.

Strichartz, G. R., T. Rando, and G. K. Wang. 1987. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* 10:236–267.

Taylor, R. E. 1959. Effect of procaine on electrical properties of squid axon membrane. *Am. J. Physiol.* 196:1071–1078.

Wang, G. K. 1984. Irreversible modification of sodium channel inactivation in toad myelinated nerve fibres by the oxidant chloramine-T. *J. Physiol. (Lond.).* 346:127–141.

Wand, G. K., M. S. Brodwick, D. C. Eaton, and G. R. Strichartz. 1987. Inhibition of sodium currents by local anesthetics in chloramine-T-treated squid axons. *J. Gen. Physiol.* 89:645–667.

Yeh, J. Z., and J. Tanguy. 1985. Na channel activation gate modulates slow recovery from use-dependent block by local anesthetics in squid giant axons. *Biophys. J.* 47:685–694.