LOCAL ANESTHETICS

EFFECT OF pH ON USE-DEPENDENT BLOCK OF SODIUM CHANNELS IN FROG MUSCLE

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ABSTRACT Sodium currents were studied under voltage clamp in the presence of neutral, amine, and quaternary local anesthetic compounds. Use-dependent block was observed as a cumulative depression of I_{Na} seen with repetitive depolarizing test pulses applied at frequencies of 2-10 s^{-1}. With quaternary QX-314, the time constant of use dependence was long, and with neutral benzocaine, very short. With lidocaine and procaine, increasing external pH (pH_o) changed the time constant from long to short, but alterations of internal pH have no effect. Inactivation in Na channels was measured by the influence of prepulses on peak I_{Na} during test pulses. Single-stimulus inactivation curves were shifted more with lidocaine at high pH_o than at low pH_o, but inactivation curves measured during pulse trains with any of the drugs and at any pH_o were strongly shifted. All measurements show that the drug-receptor reaction was slow for amine drugs at low pH_o, as for quaternary drugs at any pH_o, and fast for amine drugs at high pH_o, as for neutral drugs at any pH_o. The major effect of low pH_o on amine drugs was to reduce the concentration of drugs in the fiber and to protonate drug molecules on the receptor, thus trapping them in the blocking position for a longer time. Direct effects of pH on the receptor seemed minimal.

INTRODUCTION

Local anesthetics depress electrical excitability by blocking Na channels (Taylor, 1959) in a manner that may involve the normal inactivation gating mechanism (Weidmann, 1955; Khodorov et al., 1974). In myelinated nerve fibers, the block is enhanced by prolonged depolarization or by repetitive depolarization, and at least part of the block is removed by prolonged hyperpolarization (Khodorov et al., 1974, 1976; Strichartz, 1973; Courtney, 1974, 1975; Hille, 1977*b*). In squid giant axon, a similar cumulative effect of repetitive stimulation in the presence of some drugs can be abolished by destroying the sodium inactivation process with internal pronase (Cahalan and Shapiro, 1976; Yeh and Narahashi, 1976; Almers and Cahalan, 1977). Various aspects of this modulation by voltage pulses have been called "voltage-sensitive inhibition" (Strichartz, 1973), "slow sodium inactivation" (Khodorov et al., 1974, 1976), "use-dependent block," and "frequency-dependent block" (Courtney, 1974, 1975), but all of these manifestations may reflect the same underlying event: a slow voltage-dependent binding and unbinding of anesthetic molecules to part of the inactivation gating mechanism

of Na channels. In this hypothesis, Na channels with their inactivation gates closed bind drugs more strongly than those with inactivation gates open, so the steep voltage dependence of normal sodium inactivation gives rise to an apparent steep voltage dependence of the drug-binding equilibrium.

Our paper examines the effect of pH on the rates of binding and unbinding of local anesthetics to their receptor. Typical local anesthetics are tertiary amines which ionize with pK_a's in the range 7.5–9.0 to give an equilibrium mixture of protonated cations and neutral free amine molecules. The cation predominates at low pH, and the amine at high pH. A large literature debates which of the two anesthetic forms is active and whether the anesthetic receptor is on the internal or external face of the membrane. Permanently charged *N*-methyl or *N*-ethyl derivatives of tertiary amine anesthetics definitely do not reach their receptor when applied to the outside of an axon, but block potently when applied to the inside (Frazier et al., 1970; Strichartz, 1973; Hille, 1977*a*, *b*). The drug-receptor reaction with these cations takes place only when the Na channel is open, as if quaternary drugs must pass through the gates at the inner mouth of the channel to reach a binding site within the channel. On the other hand, amine or permanently neutral local anesthetics act rapidly when applied outside, probably because they diffuse freely through the cell membrane in the neutral form (Hille, 1977*a*, *b*). The more lipophilic drug molecules have access to the receptor at any time and show little extra accumulation during repetitive stimulation because they leave the receptor rapidly during the interval between pulses. Less hydrophobic drug forms may move only slowly in the hydrophobic pathway between pulses and must wait for the infrequent opening of the channel to provide a hydrophilic pathway. They show larger cumulative effects of repetitive stimulation.

Our study was motivated by a new observation (Khodorov et al., 1976) that recovery from slow sodium inactivation with procaine and trimecaine is slowed by lowering the external pH and speeded by raising the external pH around a myelinated nerve fiber. We tested (*a*) whether the pH change has its effect by changing the degree of ionization of the drug, the state of the receptor, or both, and (b) whether the extracellular pH is the important variable or if instead a secondary intracellular pH change brought on by the extracellular one was more important. This was achieved by analyzing the influence of pH on a use-dependent block with ionizable amine anesthetics and also permanently charged QX-314 (*N*-ethyl lidocaine) and permanently neutral benzocaine. The results show both that the change in kinetics of slow sodium inactivation comes from a change of the degree of ionization of the drug and that external pH is more important than internal. Evidently, both neutral and protonated forms of lidocaine and and procaine block Na channels. The block with the neutral form resembles the block with benzocaine and the block with the charged form resembles the block with QX-314. We present a model with only one receptor in the channel for all forms of local anesthetics and add the new hypothesis that external protons can pass through the outer mouth of the channel to alter the state of ionization of amine drugs on the receptor. All experiments were done in frog skeletal muscle fibers, where it was possible to study intracellular pH as well as to measure ionic currents under voltage clamp. A

short report of part of this work has been given to the Biophysical Society (Schwarz et al., 1977).

METHODS

Single muscle fibers were dissected and voltage-clamped by the methods of Hille and Campbell (1976) with small modifications. Fragments of twitch fibers from the semitendinosus muscle of *Rana pipiens* or *Rana temporaria* were removed in a standard Ringer's solution at pH 7.4 and placed immediately in the plastic chamber in one of the buffered "internal" solutions described in Table I. After the fiber was mounted, the ends were recut in the internal solution. The mounted fiber was then placed in the voltage clamp apparatus, and the solution in the central test compartment only was exchanged for one of the "external" solutions described in Table I. The external solutions were K-free to avoid the possible effect of K^+ ions on slow sodium inactivation (Khodorov et al., 1976), and well buffered to avoid any doubt that the pH is changed at the muscle surface. Drugs were applied dissolved in the same solutions. The drugs used were lidocaine-HCl; its *N*-ethyl derivative QX-314 bromide (both kind gifts of Dr. Bertil Takman, Astra Pharmaceutical Products, Inc., Worcester, Mass.); procaine-HCl; and benzocaine. The volume of the test pool was 150 μl. Solutions were changed by washing the test pool with 2–5 ml of drug-containing solution or 4–10 ml of control solution. No experimental records were taken before 15 min after making the second cut in the internal solutions, and most records were made at least 25 min after this cut to allow time for the ions of the internal solution to diffuse down the fiber axis to the membrane under study. The mean diameter of the mounted fibers was 135 μm in the test pool. The mean width of the recording pool was 100 μm, the Vaseline seals 200 μm, and the ground pool 140 μm. 200–700 μm of the fiber remained in each end pool after the ends were cut a second time. All experiments were done at 12.5°C.

The voltage clamp technique was improved over that described by Hille and Campbell (1976).

TABLE I

COMPOSITION OF CONTROL SOLUTIONS

External solution	NaCl	$CaCl_2$	pH	Total buffer	Buffer
	mM	*mM*		*mM*	*mM*
E6	110	2	6.0	7	2 MES + 5 Bis-Tris
E7	115	2	7.2	4	4 MOPS + NaOH
E8	110	2	8.0	10	10 tricine + TMA-OH
E9	110	2	8.8	10	10 tricine + TMA-OH
Internal solution	CsF				
I6P	60		6.0	57	8 KH_2PO_4 + 49 H_2KPO_4
I6M	60		5.9	100	100 MES + TMA-OH
I7P	115		6.9	2.4	2.2 KH_2PO_4 + 0.2 K_2HPO_4
I9P	60		9.0	40	29.6 KH_2PO_4 + 0.4 K_2HPO_4
I9T	60		9.1	100	100 tricine + KOH

Test solutions were obtained by adding the required drug to the corresponding control solution. pH values were determined at room temperature.

Abbreviations: MES, 2(*N*-morpholino) ethane sulfonic acid; bis-Tris, bis-(2-hydroxyethyl)imino-tris(hydroxyethyl)-imino-tris (hydroxymethyl) methane; MOPS, morpholinopropane sulfonic acid; tricine, *N*-tris (hydroxymethyl)-methyl glycine; TMA, tetramethylammonium.

The absolute membrane current was always obtained directly as the voltage drop across a 10 kΩ resistor rather than by attempting to "correct" the voltage applied to the current-delivery pool for the fiber impedance. This change made it unnecessary to consider any possible changes in myoplasmic conductivity after exposure to new "internal" solutions in calibrating the current records. The quality of the voltage clamp was also improved by using the same absolute current record in the adjustable correction for the effects of series resistance. The series resistance compensation was turned up as high as was practical without bringing the voltage clamp to instability. Leakage and capacity current were subtracted on-line from the membrane current with an analog circuit. The remaining current signal, representing primarily current in Na channels, was filtered with an active, four-pole, low-pass filter at 11 or 15 kHz and sampled every 30 μs by an LM[2] minicomputer kindly built for us by Dr. T. H. Kehl and his staff. Samples of both current and voltage signals were recorded on digital magnetic tape to be analyzed later. The off-line analysis, done on the same computer, involved a final correction for residual capacity and leakage currents and a determination of the peak of the inward sodium current in each record. Fibers cut in the types of internal solutions we used (containing 60 mM CsF) show no delayed currents in K channels, so it was not necessary to correct for K currents.

The potentials given are on the absolute scale (inside minus outside). Changing the external pH (pH_o) in the range from pH 6 to pH 8.8 shifted the measured voltage dependence of ordinary inactivation of Na channels by 20 mV in the hyperpolarizing direction. To compensate in part for this shift, the holding potential E_H was typically set to −80 mV for pH_o = 6, and increased to −90 or −100 mV for pH_o = 7.2–8.8. The average value of resting inactivation was then 0.73 at pH_o = 6 and 0.60 at pH_o = 8.8. Sodium currents were elicited by depolarizing test pulses lasting 1.5–2.4 ms, sometimes preceded by 50 ms hyperpolarizing or depolarizing prepulses to condition the inactivation system. The test pulses used were just slightly more positive than the value which gave the maximum inward sodium current. Use-dependent block of Na channels was observed by applying steady trains of test pulses at frequencies of 0.3-10 Hz. In each case, the peak sodium current was the major variable measured.

RESULTS

The object of the experiments is to examine the effect of pH on the interaction of local anesthetics with their receptor. We start with the effect of external pH (pH_o) changes.

Use-Dependent Block is Favored by Drug Cations

Local anesthetics block Na channels, but the degree of blocking by a given drug concentration is not a constant. Instead, block may increase or decrease upon application of different trains of voltage clamp pulses (Strichartz, 1973; Courtney, 1974, 1975). We studied use-dependent block by a procedure introduced by Courtney (1974, 1975) and used by Hille (1977*b*), involving changes of peak Na current (I_{Na}) when hyperpolarizing prepulses are turned on or off in the middle of a train of fixed depolarizing test pulses (see inset, Fig. 1). With such a pulse train, the peak I_{Na} of a fiber with only normal Na inactivation should have but two values: a high value for test pulses preceded by a prepulse and a lower value for test pulses without a prepulse. On the other hand, if there is slow Na inactivation or use-dependent block, the peak I_{Na} would not reach a stationary value in the first pulse after the prepulse is changed, but may continue to drift for several pulses, reflecting a slow development or recovery from some additional inactivated state.

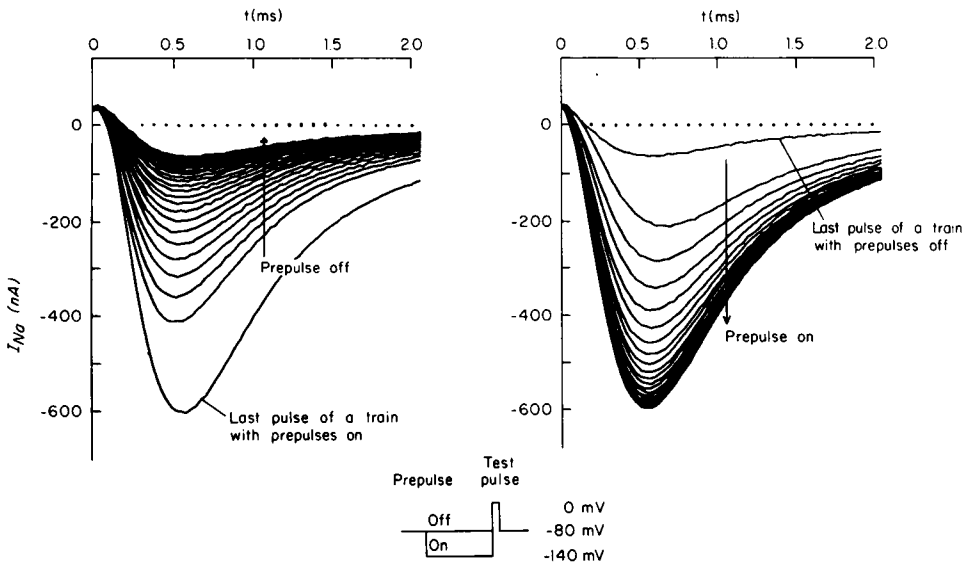


FIGURE 1 Use-dependent block and unblock of Na channels at $pH_o = 6$ with 0.2 mM lidocaine. A continuous train of 2.4-ms test pulses to 0 mV is applied at a frequency of 4 s^{-1}. Holding potential −80 mV. (A) The muscle has been conditioned by the train of test pulses preceded by hyperpolarizing pulses of 50 ms to −140 mV, and the Na currents with lidocaine are only 35% of the control value. When the prepulses are turned off, the current is smaller in the next test pulse and continues to diminish in the 25 subsequent test pulses until the current falls to only 5% of the control value without lidocaine. The time to peak is reduced in the first pulse and gradually increases to its original value during the following pulses. (B) After the train without prepulses, the hyperpolarizing pulses are turned on again. Na currents gradually regain their original value during the train. The time to peak is increased during the first pulse with prepulse and gradually decreases to its original value. Internal solution I6P, T = 12.5°C; currents filtered at 15 kHz, with leak and capacity corrected.

Development of and recovery from use-dependent block are quite obvious in muscle fibers bathed externally with 0.2 mM lidocaine at pH 6. In Fig. 1, sodium currents were elicited by 2.4-ms test pulses to 0 mV applied every 250 ms. Before the first trace given, the fiber was conditioned by a train of test pulses preceded by 50-ms prepulses to −140 mV (see inset), and the peak I_{Na} attained a value of about 35% of the control I_{Na} in the absence of lidocaine. Then (Fig. 1 A), the prepulse was removed and over the next 25 successive sweeps peak I_{Na} decreased progressively to only 5% of the value without lidocaine. Finally (Fig. 1 B), the prepulse was turned on again and I_{Na} gradually regained the previous conditioned value. We interpret these extensive slow changes as stimulus-dependent, cumulative increases or decreases in the fraction of Na channels with an occupied local anesthetic receptor. Cumulative changes requiring *several* stimuli to develop will be called "use-dependent block." In addition to gradual changes of amplitude, Fig. 1 also shows small changes in the time-course of I_{Na}. Immediately after removing the prepulse (Fig. 1 A), the time to peak is shorter than before and then gradually returns to its original value; and immediately after restoring the

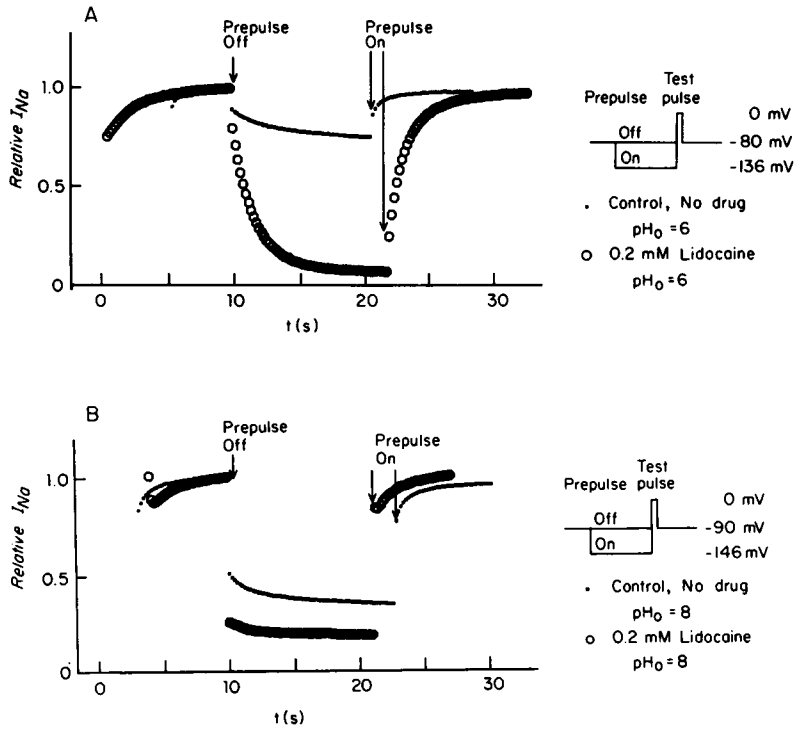


FIGURE 2 Time-course of use-dependent block of peak Na currents with and without 0.2 mM lidocaine in the external solution. Trains of 1.5-ms test pulses to 0 mV are applied at a frequency of 5 s^{-1} and hyperpolarizing prepulses are turned off and on during the train. All currents are normalized to the peak current after a train of pulses with prepulse. (A) pH_o = 6, holding potential = −80 mV, prepulse to −136 mV, maximum current with drug is 50% of value without drug. (B) A different fiber, pH_o = 8, holding potential = −90 mV, prepulse to −146 mV, maximum current with drug is 29% of value without drug. Internal solutions I6M. T = 12.5°C.

prepulse (Fig. 1 B), the time to peak is longer and then gradually returns to its original value. These small relaxations in the time to peak which require several pulses to reach completion are characteristic of all our experiments with lidocaine and procaine at low pH and are not seen before drug treatment. Courtney (1974, 1975) successfully interpreted the gradual changes in time to peak in terms of entry or exit of drug molecules during the development of the sodium current. With nodes of Ranvier bathed in 0.5–0.9 mM of the lidocaine analogue GEA-968 at pH 7.6 and stimulated every 500 ms, he found changes of amplitude and of time-course of I_{Na} similar to those we see in lidocaine at pH 6.

The time-course of use-dependent block in another experiment with lidocaine is shown in Fig. 2. Pulses were applied every 200 ms. The symbols plot normalized peak I_{Na} during a pulse train with the prepulse first on, then off, and finally on again (see inset). The small points represent control I_{Na} before treatment with drug, and the open circles, I_{Na} in the presence of 0.2 mM lidocaine, but scaled up by a factor of 2 in A and a factor of 3.4 in B to make them superimpose on the control points just before the pre-

pulse is turned off. Both control runs (points) show a step in peak current at the two changes of prepulse and an additional 10–20% slow drift during subsequent pulses. The step reflects the well-known effect of prepulses on normal Na inactivation, and the slow drift is evidence for a small component of slow Na inactivation even without added drugs (and in a K^+-free solution). This small, slow drift was found in all control runs and is more noticeable in muscle preparations than with the node of Ranvier. As was seen in Fig. 1, treatment with lidocaine at pH 6 (circles, Fig. 2 A) reduces I_{Na} and introduces a new and powerful use dependence capable of slowly blocking almost all free Na channels once the prepulse is removed. This cumulative effect of stimulation is, however, quite pH-dependent and goes away when the external pH is raised to 8 (circles, Fig. 2B), leaving simply an immediate strong increase of block when the prepulse is removed. Additionally, at high pH_o the time to peak I_{Na} does not change after the prepulse is turned on or off. Experiments like that of Fig. 2, including a pH change from 6 to 8 or from 6 to 8.8, have been repeated on 10 other fibers treated with 0.2 mM lidocaine and 10 fibers treated with 0.2 mM procaine with essentially the same results. Both lidocaine and procaine give a dramatic use dependence at pH 6 which nearly vanishes if pH_o is raised to 8.0 or 8.8. The effect of changing pH_o is rapid and reversible. No difference was seen in the results if measurements were made at 3 min or 15 min after changing to a new external pH or if the pH was first low, then high or first high, then low.

In contrast to the results obtained with the ionizable molecules lidocaine or procaine, use dependence with permanently neutral benzocaine or permanently charged QX-314 (*N*-ethyl lidocaine) is not very sensitive to changes of external pH.

Fig. 3 shows control and test runs at pH 6 and pH 8.8 compared for fibers exposed to external 1 mM benzocaine or 0.2 mM lidocaine, or to 1 mM QX-314 at the cut ends. As before, the currents in the presence of drugs were smaller than before drug treatment, but they have been scaled up to compensate for this difference (see legend). In the top panel (Fig. 3 A), the control run shows some use dependence, decreased by raising the external pH. Adding benzocaine may slightly enhance the use dependence seen in the recovery phase, but the effect is small and independent of pH. In addition, the block after removing the prepulse becomes so strong that almost no measurable I_{Na} remains. Qualitatively similar results were obtained with six other fibers exposed to benzocaine. In the bottom panel (Fig. 3C), the control run again shows a small use dependence, but 23–28 min after the fiber ends are recut in QX-314, the cumulative effect of stimulation has become enormous. Use dependence with QX-314 is equally evident at pH 6 and pH 8.8. Seven other fibers treated with internal QX-314 showed the same large, pH-insensitive effect. In conclusion, benzocaine showed little use dependence and QX-314 showed much use dependence, and the effect of both drugs are relatively insensitive to pH_o. For comparison, the middle panel of Fig. 3 shows again (in yet a different fiber from those in Figs. 1 or 2) that lidocaine-treated fibers have a pronounced use dependence when the external pH is low and almost none when the external pH is high.

The experiments with benzocaine and QX-314 suggest that an important variable governing the appearance of use dependence is the charge of the drug: a quaternary

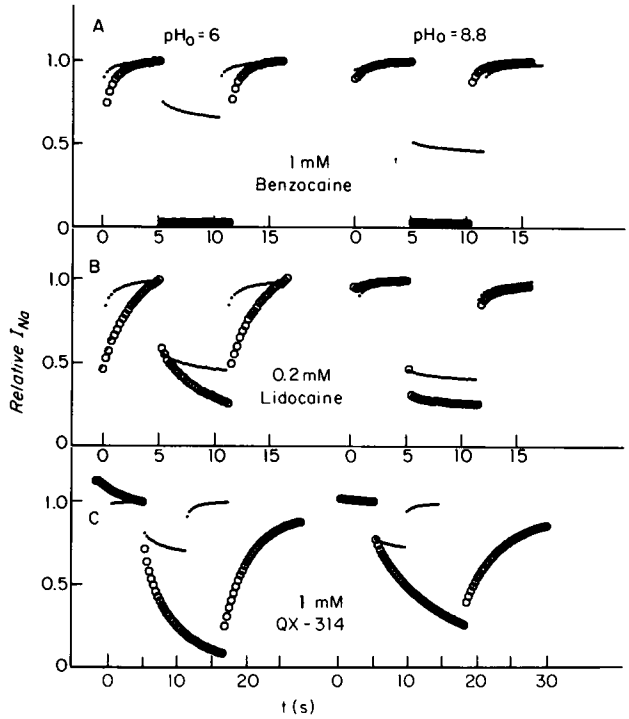


FIGURE 3 Time-course of use-dependent block of peak Na currents with benzocaine or lidocaine in the external solution or QX-314 in the solution bathing the cut ends of the fiber. The pulse train, removal and application of prepulses, and normalization of peak currents for plotting are similar to Fig. 2. Records with (open circles) and without (dots) drug are shown for $pH_o = 6$ (holding potential = −80 mV) and $pH_o = 8.8$ (holding potential = −100 mV). Pulse frequency is $4\ s^{-1}$ in A and B and $2.5\ s^{-1}$ in C. Prepulses are to −140 mV. A, B, and C are from different fibers. Reading from left to right and top to bottom, currents of 1.0 with drug correspond to the following percentage of the current without drug: 33%, 78%, 20%, 55%, 16%, and 44%. Internal solutions I9P. $T = 12.5°C$.

drug gives much use dependence, and a neutral drug does not. The same conclusion follows from the work with lidocaine and procaine where low external pH produces drug cations and much use dependence, while high external pH produces the neutral drug form and little use dependence. Evidently, both the charged and the neutral form of local anesthetics and their analogues block Na channels, but only the charged form gives use dependence. If changing the external pH also has effects on the drug receptor, they are small.

Drug Charge Affects the Rates of the Drug-Receptor Reaction

In the model developed by Courtney (1974, 1975), use dependence reflects a disequilibration of the drug-receptor interaction during stimulation. During the test waveform, the binding equilibrium is altered, and some extra binding or unbinding of drug occurs. Then, during the interval at the resting potential, there is a slow recovery towards the original equilibrium. If the recovery interval is too short for full recovery,

the effect of the next test waveform adds to the previous one, and the use-dependent disequilibrium grows. The rate and magnitude of use-dependent binding of drugs is governed by at least five factors: (*a*) the duration of each test waveform, (*b*) the duration of the recovery intervals, (*c*) the degree of difference between the binding equilibrium at rest and during the test waveform, (*d*) the kinetics of approach to equilibrium during a test waveform, and (*e*) the kinetics of approach to equilibrium during a recovery interval. In this section and the next, we give evidence that raising the external pH speeds the kinetics of the lidocaine-receptor reactions underlying factors *d* and *e*, without an important change of the equilibrium difference referred to in factor *c*.

The rate of block was studied in fibers equilibrated in 0.2 mM lidocaine solutions buffered to pH 6, 7.2, 8, and 8.8. Trains of 15–100 pulses were applied after permitting the fiber to rest at least 30 s from any previous run. The interval between pulses was changed from run to run. The period of polarization away from the holding potential was minimized by shortening the test pulses to 1.5 ms and by using no pre-

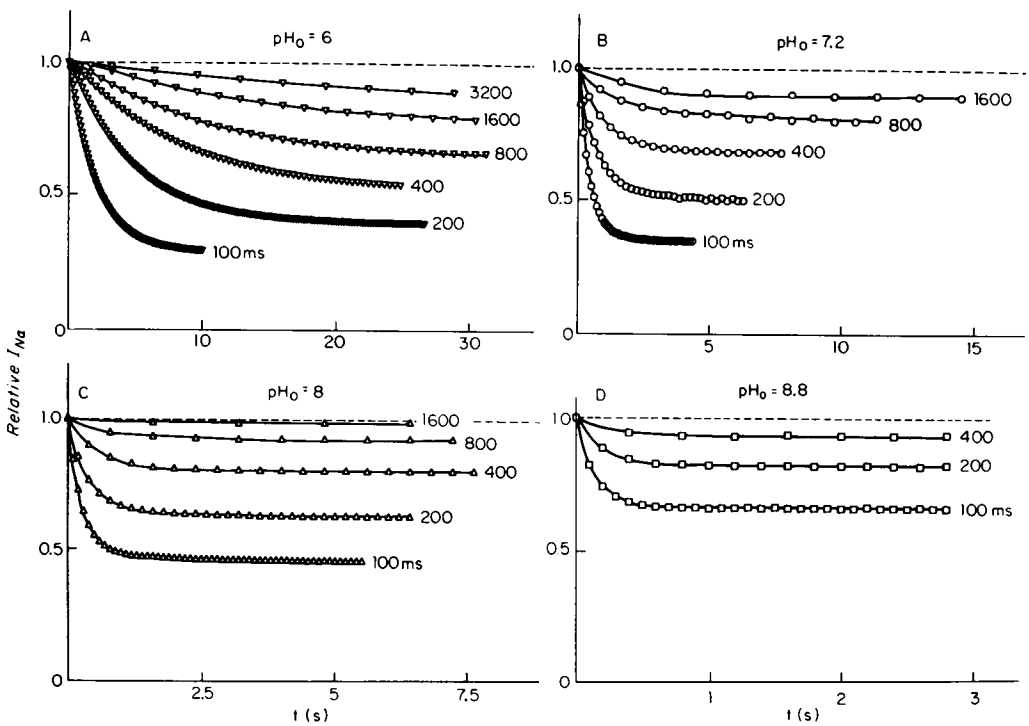


FIGURE 4 Use-dependent block of peak Na currents with 0.2 mM lidocaine for different rates of stimulation with external pH$_o$ = 6, 7.2, 8, and 8.8. Fibers were rested for 30 s at high pH and at least 120 s at low pH, and then trains of 1.5-ms depolarizing test pulses to −10 mV are applied. The numbers at each curve indicate the time interval in milliseconds between two pulses of a train. All currents are normalized to the peak current during the first pulse of a train; these currents are about 50% of the control value without drugs. Holding potential = −100 mV, internal solution 17P, T = 12.5°C; A, B, and C are from the same fiber, D is from a different fiber.

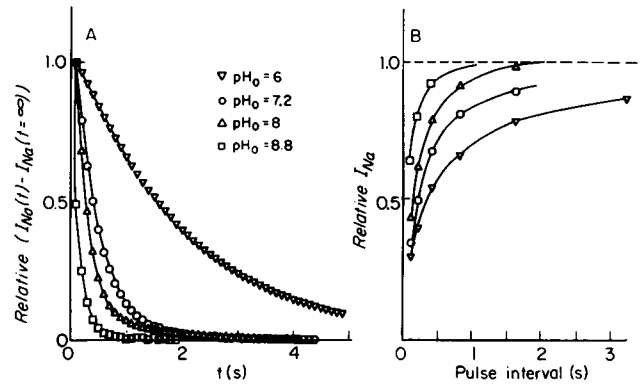


FIGURE 5 pH-dependent kinetics of use-dependent block with lidocaine. Replot of the observations in Fig. 4. (A) The time-course of the normalized difference between initial and final peak Na currents during a stimulus train with 100-ms interpulse intervals. (B) Final peak currents at the end of the pulse train plotted versus pulse interval.

pulse. Fig. 4 plots the peak I_{Na} in each pulse as a function of time for trains, with pulse intervals ranging from 100 to 3,200 ms. As before, the drug has already blocked some Na channels (40–60%) and the currents have been scaled so this resting block is not seen. The figure shows that stimulation at high frequencies increases the block more and sooner than stimulation at low frequencies, and raising the external pH speeds the development of the block as well (note the different time scales). The kinetics of the block with 100-ms pulse intervals are redrawn in Fig. 5A, which shows the time-course of the difference between peak I_{Na} and the final value of peak I_{Na} at the end of the train. None of the four curves is a single exponential, but with the criterion of time to half final block, the rates of blocking are in the proportion 1:5:9:14 at pH 6, 7.2, 8, and 8.8, with a half-time of 1.5 s at pH 6. Fig. 5B gives the final level of I_{Na} at the end of each train plotted against the pulse interval in the same experiment. At each stimulus frequency, the final block is largest at pH 6 and smallest at pH 8.8, but the effects of pH seem to be partly compensable by increasing the frequency of stimulation as the pH is raised. For example, 20% of extra block is induced with 1,600-ms interpulse intervals at pH 6 and with 400-ms intervals at pH 8. According to a model given in the Discussion section, lowering the external pH slows the rate of departure of drugs during the recovery interval by increasing the degree of protonation of neutral drug molecules bound to the receptor, and decreases the rate of drug entry during the test pulse by decreasing the concentration of drugs in the membrane and in the cytoplasm.

Drug Charge Has Little Effect on the Interaction with Inactivation Gates

Our experiments on the kinetics of a use-dependent block were done with pulses that favor a strong increase of block over that at the holding potential. As Figs. 1–3 have shown, repetitive stimulation can also give recovery from block if each test pulse is preceded by a sufficiently negative hyperpolarizing prepulse. When Courtney (1974,

1975) found this unblocking effect, he proposed that the voltage dependence of the inactivation gating mechanism in a Na channel becomes shifted to more negative potentials when a local anesthetic molecule blocks the channel. From an equilibrium thermodynamic viewpoint, a shift of inactivation with binding necessarily means that the drug binds more strongly to channels with closed inactivation gates than to channels with open inactivation gates. Hence, large hyperpolarizing prepulses weaken drug binding by opening inactivation gates, and then more drug leaves the channels than enters.

Depending on the technique used for measurement, at least three different "steady-state inactivation" curves can be measured in drug-treated fibers. All methods measure the peak I_{Na} during a fixed test pulse after some conditioning period. The first technique is the conventional two-pulse method of Hodgkin and Huxley (1952), where the conditioning is a single prepulse 10–100 ms in length and the pulses are applied so infrequently that there is no cumulative effect from one test pulse to the next. In the second technique, the conditioning is a rapid train of prepulse-test pairs applied until a steady state of use dependence is reached (Courtney 1974, 1975). In the final method, the conditioning is a prepulse lasting a second or more or a change to a new holding potential (Khodorov et al., 1974, 1976; Hille, 1977*b*). In this paper we reserve the term "h_∞ curve" to describe the voltage dependence of the inactivation gating mechanism of the channel, and use the terms "inactivation measurement" and "inactivation curve" operationally to describe the result of any of the three types of experiments. Under conditions where the time constants for drug binding and unbinding are long, the Hodgkin-Huxley technique might be expected to measure the conventional h_∞ curve of those channels not blocked by the drug at rest, while the second and third techniques, which involve repetitive or long polarization, should also include effects of binding and unbinding of the drug and reflect in part the h_∞ curve of blocked channels. When the time constants for drug binding and unbinding are short, all three methods will reflect a combination of the inactivation gating processes and the drug reactions.

Fig. 6 shows inactivation measurements of the first and second kind under conditions where the drug-receptor reactions are expected to be either slow or rapid. In each frame the points represent observed peak currents without any separate scaling of the control and drug-treated cases. Control inactivation measurements are shown as filled symbols fitted by smooth curves, and measurements with a drug as open symbols, together with control curves scaled down by factors given in the legend. As anticipated for a slow drug-receptor reaction, external lidocaine at pH 6 or internal QX-314 reduce I_{Na} but do not appreciably change the shape of the inactivation curve measured with single test pulses (circles, Fig. 6A and C). Evidently, the anesthetic molecules do not change inactivation in those channels not blocked by the drug, suggesting that drugs act at a specific receptor rather than by dissolving diffusely in the membrane. Trains of pulses (triangles, Fig. 6A and C), however, produce considerable negative voltage shifts of the midpoints of the inactivation curves and decreases of its slope. These arise as the block is gradually relieved by extremely negative prepulses

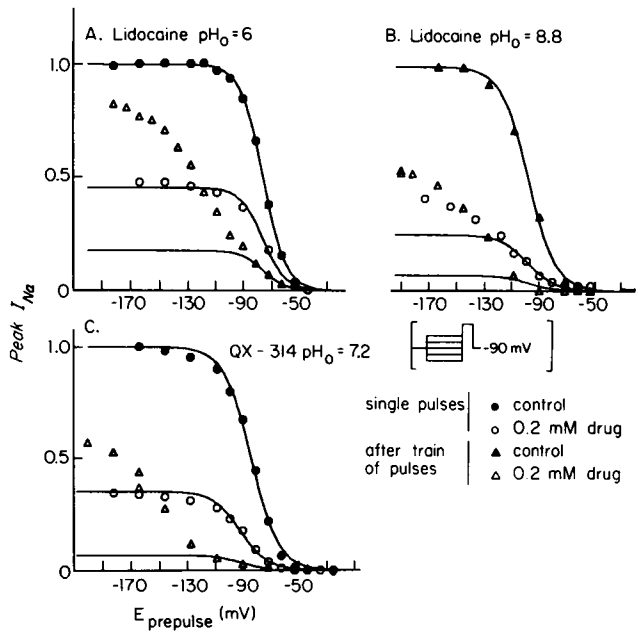


FIGURE 6 Two kinds of inactivation measurements with and without drugs. Effect of prepulse potential on peak Na currents with 0.2 mM lidocaine at pH_o = 6 and 8.8, and with 0.2 mM QX-314 at the cut fiber ends with pH_o = 7.2. Depolarizing test pulses of 1.5 ms to 0 mV were preceded by 50-ms prepulses to different potentials from a holding potential of −90 mV. A, B, and C represent different fibers. All currents are given relative to the maximum control values without drugs (filled symbols). Solid lines through filled circles are least-squares fits of $1/(1 + \exp[(E - E_h)/k_h])$. The same curves are repeated, scaled down by factors of 0.45 and 0.18 in A, 0.25 and 0.07 in B, and 0.35 and 0.07 in C. Open symbols represent peak currents during drug treatment: circles, determined with single pulses on fibers rested at least 70 s between records; triangles, final peak currents after a train of pulses at a frequency of 10 s^{-1}. Internal solution 17P. T = 12.5°C.

and enhanced by prepulses closer to the resting potential. We see this new curve as reflecting more closely the inactivation gating process in blocked channels. In this interpretation, QX-314 and the protonated cationic form of lidocaine shift the voltage dependence of h_∞ in blocked channels by 40–55 mV and reduce the slope of the h_∞ curve by up to 35%. With lidocaine at pH 8.8, where the drug-receptor reactions are faster (Fig. 6B), the differences between the two kinds of inactivation measurement are much less pronounced, and a shift of inactivation relative to the control is apparent even with single test pulses. Similarly, with neutral benzocaine, the inactivation curve measured with single test pulses is also very shifted, as is apparent from the nearly complete disappearance of sodium current when the prepulse is removed, in Fig. 3A for example. In one experiment with 1 mM benzocaine the midpoint of the inactivation curve was shifted by 29 mV, the slope factor (k_h) changed from 8.4 to 11.4 mV, and the current with the most negative prepulses was reduced to 55% of the control. Evidently, there is a large change of the h_∞ curve in all channels that have a bound anesthetic molecule, whether the bound molecule is neutral or cationic.

External pH Changes Have Little Effect on Internal pH

We turn now to consider whether the important pH change in these experiments is the applied external change or a secondary internal change. The effect of external bathing pH on the intracellular pH was studied with whole semitendinosus muscles soaked in strongly buffered pH 6 and pH 8.8 external solutions (E6 and E8, Table I). After the desired time, the muscles were transferred to a solution at the same pH but with only 1 mM buffer for 2 min, and finally blotted and homogenized in distilled water. In one series of single determinations at room temperature, individual muscles exposed to pH 6 Ringer for 15, 33, and 240 min had homogenate pH values of 7.2, 7.1, and 6.8, and muscles exposed to pH 8.8 Ringer for 23, 50, and 253 min had pH values of 7.04, 7.02, and 7.09. In another similar series at 12°C, the homogenate pH values changed even less. These tests show that the internal pH (pH_i) is hardly influenced by the external bathing pH during the typical 60-min duration of the voltage clamp experiments already described, and the change of pH_i must be far less than 0.1 pH unit when anesthetic effects are measured first at one external pH and then 5 min later at another. For comparison, direct measurements with pH-sensitive glass microelectrodes in sheep heart Purkinje tissue showed an 0.1 pH unit drop of pH_i 14 min after the extracellular pH was reduced from 7.4 to 6.4 (Ellis and Thomas, 1976).

Internal pH Changes Are Not the Variable Affecting Use Dependence

The state of ionization of intracellular amine anesthetic would necessarily depend on the internal pH. To study its influence on use-dependent block, we changed the internal pH by cutting the ends of fibers in various buffered "internal solutions" (Table I). The resulting values of pH_i were not measured directly but rather were calculated by a procedure described in the Appendix. These calculated values are termed "predicted" in this section. Changing pH_i seemed to have only minor effects on the sodium currents either in the absence or in the presence of local anesthetics. We did not make a full and careful study of the kinetics of I_{Na}, but in the few spot checks done, there was no major block of I_{Na} or change of the kinetics in the 45 min after cutting the ends of fibers in solutions with 100 mM buffers at pH 9.1 or pH 6 (predicted pH_i = 8.8 or 6.3 at 45 min). More interestingly, there was little change of the use dependence seen with lidocaine, as may be seen by comparing the experiments in Figs. 2, 3B, and 7A, recorded 23–25 min after cutting fibers in solutions buffered with 100 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) at pH 6 (Fig. 2), 40 mM phosphate at pH 9 (Fig. 3B), and 100 mM tricine at pH 9.1 (Fig. 7A). Although the predicted internal pH values in these fibers are 6.5, 7.3, and 8.4, the use dependence with lidocaine looks about the same in each case. At low external pH it is large and at high external pH, minimal. Evidently, external pH is the important variable affecting the appearance of use dependence with lidocaine, and internal pH has little influence. This is demonstrated again by the difference in use dependence between the experiment of Fig. 7A and that of Fig. 7B. The fiber of Fig. 7A has a predicted internal pH of 8.4, an external pH of 6, and shows strong use dependence from lidocaine, while the fiber of Fig. 7B, like that of Fig. 2B, has a predicted internal pH of 6.5, an ex-

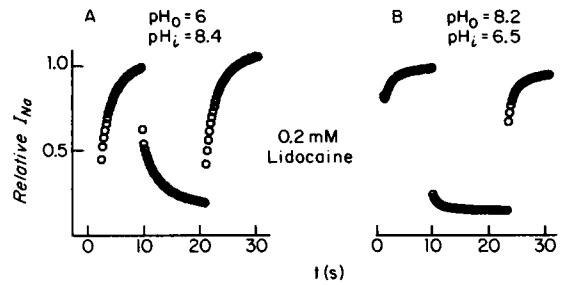


FIGURE 7 Lack of effect of internal pH on use-dependent block of peak Na currents with 0.2 mM lidocaine. Depolarizing test pulses of 1.5 ms to 0 mV were preceded by 50-ms prepulses to −140 mV. Holding potential −80 mV. (A) 26 min after cutting in internal solution I9T, giving a predicted pH_i = 8.4. Outside solution E6, with pH_o = 6. (B) 25 min after cutting in internal solution I6M, giving a predicted pH_i = 6.5, outside solutions E8, with pH_o = 8. This experiment was similar to Figs. 1, 2, and 3.

ternal pH of 8, and shows little more use dependence with lidocaine than is found in similar situations without lidocaine. A model is given in the Discussion section explaining how the external pH may be a significant variable even if the drug receptor is not accessible to cationic drug forms in the external medium.

DISCUSSION

Basic Conclusions and Comparison with Previous Work on Frog Nerve

This paper is the first voltage clamp study of the effect of local anesthetics on Na channels of skeletal muscle. As has already been concluded from other criteria (Campbell and Hille, 1976), we find that the Na channels of frog skeletal muscle are extremely similar to those of frog myelinated nerve fibers. Like Na channels in nerve (Strichartz, 1973; Århem and Frankenhaeuser, 1974; Courtney, 1974, 1975; Khodorov et al., 1974, 1976; Hille, 1977*a*,*b*), Na channels in muscle are blocked by 0.1–1.0 mM procaine, lidocaine, and benzocaine applied externally and by 0.2 mM QX-314 applied internally. At a fixed stimulus frequency, the use-dependent accumulation of drug diminishes in the sequence: quaternary drugs, amine drugs, neutral drugs. The amount of shift of the inactivation curve seen with single test pulses falls in the reverse sequence: neutral, amine, quaternary; but all drug types shift the curve by tens of millivolts and reduce its slope when tested with trains of test pulses. All of these properties are common to nerve and muscle. One of the few differences we noted between Na currents of frog muscle and those of frog nerve is that even in the absence of drug, the currents in muscle showed small, prepulse-dependent, cumulative changes during repetitive stimulation at rates as low as 2 Hz (Figs. 2–3). Similar and even more pronounced effects of repetitive stimulation without drugs have been reported in the giant axons of *Myxicola* (Rudy, 1975).

Khodorov et al. (1976) first reported that changing external pH changes the kinetics of recovery from local anesthetic-induced slow sodium inactivation. In most of their

experiments, the slow sodium inactivation was precipitated by a single long (1 s) depolarizing conditioning pulse, but they also showed that recovery from such a pulse follows the same kinetics as recovery from use-dependent block after a train of 1-ms depolarizing pulses. Thus, we believe that the blocking and recovery processes we have studied are the same as those studied by Khodorov et al., although we use different pulse patterns. Our observations confirm a slowing of the kinetics of use-dependent block by lowering external pH and a speeding up by raising external pH. These changes do not occur with neutral benzocaine or charged QX-314. Therefore, if there is only one receptor for all drug forms, the primary effect of pH must be on the drug and not on the receptor. The muscle fibers we use are so large and well buffered that their internal pH is virtually constant during the changes of extracellular pH. Furthermore, deliberate attempts to change intracellular pH did not seem to affect use dependence. Hence, pH_o is the important variable affecting drug ionization.

As the external pH is raised from a low to a high value, the effect of amine anesthetics changes from those typical of a quaternary drug cation to those typical of a neutral drug form. We believe that the changes may be understood in the following terms: (*a*) There is a single receptor for all drug forms. (*b*) Bound drugs block the the channel. (*c*) Bound drugs modify the Na inactivation gating mechanism. (*d*) Neutral drugs can come and go rapidly from the receptor while charged drugs are unable to come and go except when the channel is open. (*e*) The concentration of drugs within the fiber is profoundly affected by external pH. Finally, (*f*) we need a new hypothesis, to be justified later: external protons can equilibrate with the bound drugs through the open mouth of the channel.

Our major evidence for supposing that there is a single receptor for the two charge forms of the drug is that cationic and neutral forms block Na channels and also modify the inactivation gating process in the same way (see Fig. 6 and also Hille, 1977*b*). The modification of inactivation is not a simple shift of the voltage dependence, as might be caused by a diffuse surface charge effect, but might better be described as a decrease in the normal rate constant α_h for removal of inactivation and/or an increase in the rate constant β_h for development of inactivation. For example, if in blocked channels α_h were reduced 50-fold, the calculated h_∞ curve would have its midpoint shifted by -31 mV and its slope reduced by 30%, and the rate of recovery from inactivation at hyperpolarized potentials would be slowed considerably. A thermodynamic corollary of such a change is that anesthetics should bind 50 times better to inactivated channels than to resting ones, producing an apparent voltage-dependent binding equilibrium, as is observed. This suggested description in terms of changes of α_h or β_h is, however, entirely untested.

The effects of pH_o on use dependence and on inactivation measurements can now be given a qualitative explanation. The experiments of Figs. 1–3 show that the number of blocked channels changes with repetitive stimulation. With a hyperpolarizing prepulse on, inactivation is removed and drug unbinding is favored. With the prepulse off, most channels remain inactivated and drug binding is favored. The time constants of the resulting use dependence lengthen at low pH (Figs. 4 and 5) as more

drug acts in cationic form, while at high pH, where more drug acts in the neutral form, the time constants can be so short that no cumulative effects of stimulation are seen with frequencies up to 5 Hz. The changes of the time to peak I_{Na} in Fig. 1 at $pH_o = 6$ are a reflection of the kinetics of the extra entry or departure of drug molecules during the rising phase of I_{Na} (Courtney, 1974, 1975). Shortening of time to peak occurs with net drug binding, and lengthening with net unbinding. The perturbation of the time to peak is small, either when the extra entry per pulse is small, e.g., with QX-314, or when the rate of drug binding and unbinding are rapid enough to be near equilibrium, e.g., with amine drugs at high pH or benzocaine. The variety of changes of the inactivation curves in Fig. 6 is another manifestation of fast drug reactions at high pH_o and slow ones at low pH_o. With lidocaine at low pH_o (Fig. 6A), a conventional test of inactivation shows the same voltage dependence as without anesthetic, since the number of drug-blocked channels does not change fast enough to influence a single measurement appreciably. Repetitive stimulation then produces a use-dependent block or unblock that reveals the effect of bound drug on inactivation gating. The situation is identical for experiments with QX-314 inside (Fig. 6C). With lidocaine at high pH (Fig. 6B) or with benzocaine, the drug-receptor reaction reequilibrates during the prepulse, revealing the modified inactivation gating in one pulse and little further use-dependent change. We now develop a kinetic model to describe explicitly how pH_o affects the time constants of the binding and unbinding of drugs.

A Kinetic Model of Drug Binding and Ionization

One of the clearest conclusions of previous work with quaternary derivatives of lidocaine, trimecaine, and other local anesthetics is that there is no externally accessible binding site for such analogues and that small cationic drug forms do not cross the excitable membrane at an appreciable rate (Frazier et al., 1970; Strichartz, 1973; Khodorov et al., 1976; Hille, 1977*a*,*b*). Cationic drug forms reach the drug receptor only from the intracellular side of the membrane. By contrast, tetrodotoxin and saxitoxin cations have an externally located receptor, and, as expected, their binding is not reduced by added local anesthetics (Colquhoun et al., 1972; Henderson et al., 1973; Wagner and Ulbricht, 1976). For these reasons it is surprising to find that external pH plays a larger role than internal pH in modifying the kinetics of use-dependent block with local anesthetics. How does an increase in external protons influence drug molecules which react with the receptor?

The amount of drug in the fiber should depend strongly on the external pH. In the external solution, the fraction R of drug molecules in the neutral amine form is related to the external hydrogen ion concentration $[H^+]_o$ by the usual bimolecular formula

$$R = K_a/(K_a + [H^+]_o), \tag{1}$$

where K_a is the acid dissociation constant of the drug. At the ionic strength of our solutions, K_a is about $10^{-8.1}$ for lidocaine at 25°C (Löfgren, 1949; Robinson and Stokes, 1965). Because of the extremely high permeability of neutral amine drug (ca. 1 cm s^{-1}, Hille, 1977*a*), the concentrations of neutral drug inside and outside the cell

should become equal in a short time, making the amount of neutral amine drug in the membrane and inside the cell proportional to the function R. Finally, if the intracellular pH is constant, the concentration of intracellular cationic drug bears a constant ratio to the concentration of neutral drug and must, therefore, be proportional to R as well. Thus, the concentration of both drug forms inside the cell rises to an asymptotic value at high pH_o and falls to very low values at low pH_o. Dettbarn et al. (1972) have verified this conclusion by measuring the pH_o-dependence of the distribution of radioactive procaine between axoplasm and sea water in squid giant axons exposed to drugs for 10 min. The relative axoplasmic drug concentration was 17% for pH_o = 5.8, 91% for pH_o = 7.0, and 627% for pH_o = 9.0. Although they state that 10 min is probably not long enough to reach equilibrium in the giant axon, their pH 7.0 value is the value expected from equilibrium, and for an intracellular diffusion coefficient of 0.25×10^{-5} cm^2 s^{-1} we calculate the expected time to reach half the mean equilibrium concentration in a 700-μm fiber to be less than 1 min (Crank, 1956).

According to arguments summarized in earlier papers (Hille, 1977*a*,*b*), the drug receptor is within the Na channel and drug molecules come and go from the receptor via the membrane phase (hydrophobic pathway) and via the inner mouth of the channel (hydrophilic pathway). We have just seen that all drug forms available for reaction with the receptor (i.e., those in the membrane and those in the cell) vary in parallel as pH_o is varied. Yet the effects of pH_o on use-dependent block are clearly not just equivalent to a change in drug concentration. Therefore, we see no alternative to introducing the hypothesis that drug molecules bound to the receptor and blocking the channel can acquire or give up protons via the external mouth of the channel to the external medium. This idea is illustrated diagrammatically in Fig. 8A, which shows a drug molecule sitting on the inner side of the narrow selectivity filter of the channel.

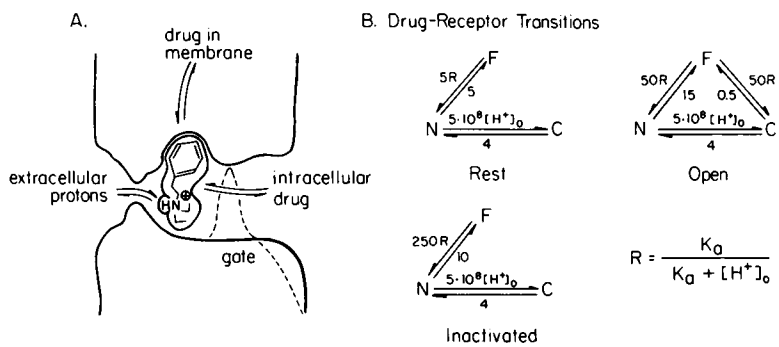
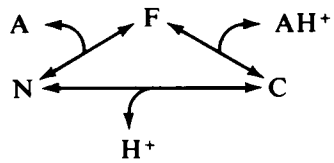


FIGURE 8 Kinetic model of drug binding and ionization. F, unblocked channel free of drugs; N, channel blocked by a neutral drug; C, channel blocked by a charged drug. The three schemes represent transitions in the resting state, during the test pulse when channels are in the open state, and after open channels have become inactivated. The numbers are rate constants used in the simulation of use-dependent block with 0.2 mM lidocaine (cf. Fig. 9) given in per second or per mole per liter per second. $R = K_a/(K_a + [H^+]_o)$, where K_a is the acid dissociation constant of drug ($K_a = 10^{-8.1}$ for lidocaine) and $[H^+]_o$ is the external molar proton concentration. The function R expresses the pH dependence of the concentration of reactive drug forms.

In effect, the external pH governs the state of ionization of bound drug molecules and affects how long they remain on the receptor. Formally, the drug receptor is in one of three states: *F*, channel free of drugs and not blocked; *N*, channel blocked by a neutral drug; and *C*, channel blocked by a cationic drug. These states interconvert by the kinetic scheme:



where A is an available free amine drug, AH^+ is an available protonated drug, and H^+ is a proton exchanged with the external medium. As is supposed for other small cations, the exchange of protons may be catalyzed by the acid group of the selectivity filter and might be weakly voltage dependent (Woodhull, 1973).

Earlier work showed that the rate and equilibrium constants of drug binding depend strongly on whether the channel is resting, open, or inactivated (Strichartz, 1973; Courtney, 1974, 1975; Khodorov et al., 1974, 1976; Hille, 1977*a*,*b*). In principle, it is then appropriate to distinguish resting, open, and inactivated forms of the F, N, and C states, giving a nine-state kinetic scheme with many steps involving Hodgkin-Huxley kinetics. However, such a scheme is more complex than needed to make a simple qualitative test of the principal ideas. For a more manageable simulation of the development of use dependence during a pulse train, we use the simpler three-state FNC model, but take into account that the transitions among states have different probabilities during conditioning pulses than at rest. Specifically, three periods are distinguished, represented by the kinetic diagrams and rate constants given in Fig. 8B. They are the rest interval and two periods associated with the depolarizing test pulse. These latter might be loosely identified with the time when channels are open and a period during and after the test pulse when channels are inactivated, but the identification is at best rough, as the model is a grossly simplified representation of the events thought to occur.

Application of the Model

Our model lumps together the processes of diffusion of drug to its receptor and the reaction with the receptor as a single first-order "binding" step. The rate constants shown in Fig. 8B were chosen by trial and error to imitate the experiment of Fig. 4, in which a train of test pulses was applied to a rested fiber with different pulse intervals and at different pH_o values. Binding rate constants are for 0.2 mM lidocaine and vary with pH_o, being proportional to the function R (Eq. 1) to account for the pH dependence of the concentration of available drug forms A and AH^+. For example, the binding of both neutral and charged drugs during the test pulse has a pseudo first-order rate constant of $50 \cdot R\ s^{-1}$. This may be converted to equivalent bimolecu-

lar rate constants of 2.5 × 10^5 M^{-1} s^{-1} for neutral and 2.5 × 10^4 M^{-1} s^{-1} for charged drug by dividing by their respective intracellular concentrations, 0.2 *R* mM and 2.0 *R* mM. For simplicity in this conversion, the "reacting" drug is assumed to come from the intracellular compartment rather than the membrane and the intracellular pH is taken as 1 pH unit below the pK_a of lidocaine (8.1), making the ratio of internal cations AH^+ to neutral particles A about 10:1. While the binding of neutral drug is permitted at all times and is accelerated 10-fold by "opening" channels and another fivefold by "inactivating" them, a direct binding or unbinding of the drug cation is not permitted when channels are closed or inactivated. Bound drug cations are effectively trapped in the channel until they lose their charge or the channel opens.

The equilibrium of bound drugs with external protons takes place any time with a forward rate constant of 5 × 10^8 M^{-1} s^{-1}, 20–50 times smaller than the diffusion-limited maximum in free solution (Eigen and Hammes, 1963), and with a reverse rate constant chosen to give a pK_a of 8.1 for the drug. The possible voltage dependence of these steps (cf. Woodhull, 1973) has been ignored. Protons should be able to enter the channel even in the absence of drugs. If the gates of the channel are open, the proton flux would create a minute membrane current on the order of 2,000 protons s^{-1}

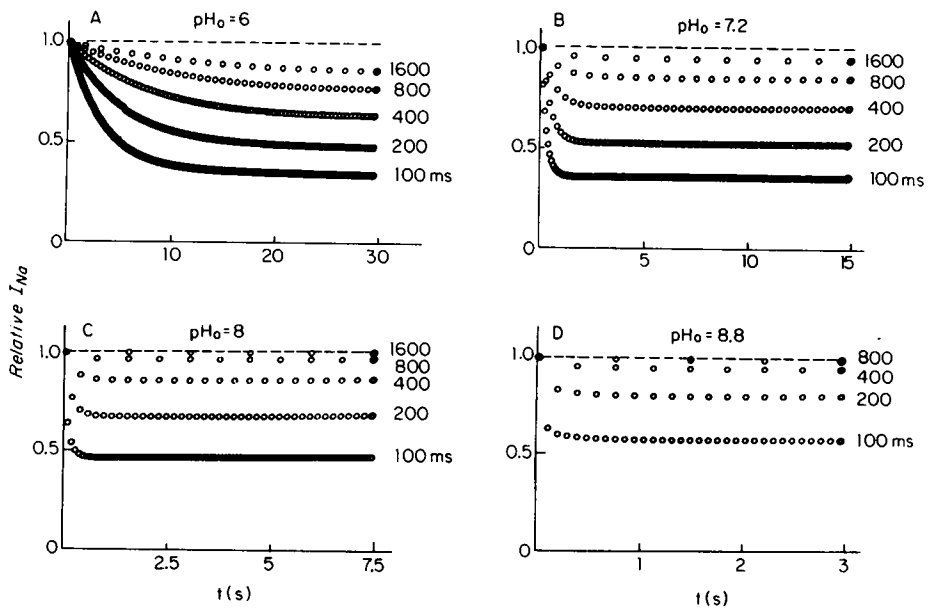


FIGURE 9 Simulation of the experiment of Fig. 4, using the model of Fig. 8. Use-dependent block of sodium peak currents for different rates of stimulation with external pH_o = 6, 7.2, 8, 8.8, and 0.2 mM lidocaine. Trains of test pulses were simulated by assuming that the channels are open for 1.5 ms, inactivated for 10 ms, and at rest until the next pulse. Numbers at the ends of the curves give the pulse intervals in milliseconds. The points are arithmetic means of unblocked channels at the beginning and end of the open state, normalized to the first point. The current in the first pulse is 50.0, 49.7, 48.6, and 47.5% of the drug-free current in A, B, C, and D, respectively.

at pH_o = 5.4, given an entry rate equivalent to the forward rate of protonation of amine drug used in our model. This probably undetectable current is entirely compatible with the assumption that proton entry is catalyzed by an acid group with pK_a of 5.4 in the selectivity filter (Woodhull, 1973).

Fig. 9 shows a simulation of use dependence with the model. The calculation starts with the proportions of F, N, and C at their equilibrium values for a rest period, and then this equilibrium is perturbed by a train of 1.5-ms "test pulses," each followed by a 10-ms period of "inactivation" and the appropriate "recovery" period. To represent more closely the number of Na channels open at the time of the peak sodium current, the points plotted are the average of the calculated amounts of free channels immediately before and immediately after each test pulse, normalized to make the first average 1.0. The calculation successfully reproduces qualitiative aspects of the observations in Figs. 4 and 5. Use dependence increases with increasing frequency of stimulation, and the time constant and depth of use dependence increase with decreasing pH. The slowing at low pH_o is caused by (*a*) a decrease in the rate of drug binding during the test pulse due to a decrease in the function *R*, and (*b*) a decrease in the rate of unbinding during recovery due to trapping of most bound drug molecules in the cationic form. For recovery from block at rest, the predicted time constant of the most important relaxation at each pH is 13, 1, 0.3, and 0.1 s at pH_o = 6, 7.2, 8, and 8.8. These values for the model at 12.5°C are more pH-dependent but still roughly comparable to values given by Khodorov et al. (1976) for recovery from slow sodium inactivation with 0.09 mM procaine at 24°C. For nodes of Ranvier at −95 mV they found time constants of 0.65, 0.3, and 0.2 s at pH_o = 5.9, 7.3, and 8.5.

There is not complete agreement about the effect of pH_o on the equilibrium potency of amine local anesthetics acting on myelinated nerve. In action potential studies, Skou (1954) found a 16-fold increase in potency of procaine as pH_o was raised from 6 to 8; Ulbricht and Walle (1976) found a fivefold increase on going from pH 6 to 7.2 and a sevenfold increase on going from 7.2 to 9; and Dettbarn (1962) found a maximum potency near pH_o = 7. In voltage clamp studies on hyperpolarized fibers, Århem and Frankenhaeuser (1974) reported a gradual threefold increase in potency of lidocaine as pH_o was raised from 6 to 10, and Khodorov et al. (1976) reported no change with procaine or trimecaine on raising pH_o from 6 to 7.3 and a small increase on raising pH_o from 7.3 to 8.5. These apparent differences might be explained by saying that the block in hyperpolarized nerve depends only weakly on pH_o and that an increase in block with increasing pH_o appears in fibers maintained at their resting potential because of the normal pH-dependent shift of sodium inactivation which increases resting inactivation and hence drug binding at high pH_o. In our model (Fig. 8B, "Rest"), equilibrium receptor occupancy actually is independent of pH_o despite the pH dependence of the reactive drug fraction *R*, since an increasing conversion of neutral drug to cation in the channel exactly compensates for the drop in *R* at low pH_o. This compensation would not be exact for cases where the pK_a of the drug is different in the channel than in free solution. For the constants given in Fig. 8B and used in Fig. 9, the equilibrium block is 50%. However, a slightly larger increment of the drug is drawn

into the channel during a single test pulse at high pH than at low pH, so the "current" measured by a single test pulse is actually slightly smaller at high pH (see values given in legend of Fig. 9).

In agreement with the hypothesis that there is only one receptor for all drug forms, the model is easily adapted to simulate experiments with benzocaine or QX-314 using the appropriate rate constants for lidocaine with minor changes. For benzocaine, only the F–N reaction of Fig. 8B is permitted since there is no charged drug form. If the forward bimolecular rate constants and the reverse first-order rate constants are taken to be the same as those for neutral lidocaine, then with 1 mM benzocaine 83% of the channels would be blocked at rest, and use-dependent block would not be evident for interpulse intervals longer than 200 ms. If future experiments with shorter interpulse intervals show no use dependence, then faster rate constants would be required. For QX-314, there would be no permitted transitions during the rest or inactivated periods, and the reaction scheme is limited to the F–C step during the test pulse. The time-course of use-dependent block in Fig. 3C is well simulated by a forward first-order rate constant of 20 s^{-1} and a reverse rate constant of 0.5 s^{-1}. Assuming a QX-314 concentration inside the cell of 0.5 mM, the equivalent forward bimolecular rate constant is 60% larger than for lidocaine cations, and the reverse rate is the same as for lidocaine cations. Finally, although the emphasis in this paper has been on the effects of drug charge, it should be noted that other molecular properties, including shape, size, and hydrophobicity also have important effects on the drug-receptor reactions. For example, Courtney (1974, 1975) showed that recovery from use-dependent block with 0.25–1.0 mM of the hydrophilic lidocaine analogue GEA-968 (pK_a = 7.7) has time constants in the range 7–15 s at pH_o = 7.6. Such slow recovery would be simulated in our model by assigning to GEA-968 binding and unbinding rate constants 50 times slower than those for lidocaine in the rest period. We suppose that such slow rates simply reflect the difficulty of the more hydrophilic GEA molecule to enter and leave the channel via the membrane phase.

The most important conclusions of this paper are that charged and neutral drug forms can both block Na channels and that they do so by acting on a single site within the channel, closely tied to the inactivation gating system and yet accessible to protons in the external medium. The qualitative evidence for these points is bolstered by a semi-quantitative agreement between model calculations and the experiments on use-dependent block. The model is recognized as an oversimplified and somewhat arbitrary representation of the underlying transitions of the drug receptor. It could be tested further by application to observations other than use dependence with lidocaine. For example, both procaine and GEA-968 give a more pH-dependent resting block than lidocaine (Århem and Frankenhaeuser, 1974; Hille, 1977*a*), and use dependence with GEA-968 extends to much lower frequencies (Courtney, 1974, 1975). The model does not deal explicitly with the kinetics of I_{Na} or with changes of inactivation curves. To do so would require reverting to the full nine-state version of the FNC scheme, including many steps with Hodgkin-Huxley kinetics. It will probably be necessary to use such models ultimately in analyzing more carefully how the inactiva-

tion gating mechanism is modified by local anesthetics. In the meantime one must be careful not to attribute overly specific physical interpretations to the individual rate constants used in the simplified model.

The influence of external pH on drug molecules bound to their receptor adds new evidence in favor of Strichartz's (1973) original suggestion that the bound drug lies in a space between internal gates and an external selectivity filter, rather than lying on the intracellular side of the gates. The action of external protons in this space probably has implications beyond the subject of local anesthesia. There may be many drugs acting within Na or K channels whose state of ionization and rate constants for unbinding can be modified by exchanging protons with the outside. Amino or carboxyl compounds, like antiarrhythmic agents, aminopyridines, and barbiturates, are possible candidates. An increase of use dependence at low tissue pH and enhanced binding of drug to depolarized cells may be important mechanisms in the antiarrhythmic actions of lidocaine, quinidine, and propranolol on diseased or ischemic cardiac tissue. Finally, even without a drug, it seems quite likely that protons exchange with the channel, and, if the gates at the inner end of the channel are closed, the pH within the channel may follow the external pH closely. Indeed, any permeant ions in the external medium might in this way occupy closed pores, leading to possible ion-specific modifications of the functioning of channels.

APPENDIX

Calculation of Internal pH Change

We change the intracellular pH by cutting the ends of mounted muscle fibers in buffered solutions. The value of pH_i in the region under voltage clamp was estimated by calculating the diffusion of buffer down the fiber axis and the buffer reactions with myoplasm. This calculation required knowing the intracellular buffer value of semitendinosus muscle. Single muscles weighing 265–545 mg were blotted, weighed, frozen in liquid nitrogen, and ground in a mortar and pestle precooled with liquid nitrogen. The resulting powder was taken up in 20 ml of distilled water as a milky suspension and titrated with 20 mM NaOh or 20 mM HCl standard solutions while the pH was monitored with a glass electrode. The entire procedure took less than 1 hr. Two titrations were done at room temperature and one at 0°C with similar results. The titrations were done with a strong stirring in air, conditions which probably cause a loss of CO_2–HCO_3^- buffers from the suspension. The mean initial pH of the freshly prepared homogenates was 7.0, close to values of 7.0–7.2 obtained by other methods for the intracellular pH of frog skeletal muscle (see references in Izutsu, 1972). The titration curve of one muscle is shown by triangles in Fig. 10. Averaged buffer values calculated from the three titrations are shown as squares in the same figure. At pH 7.2, the estimated buffer value is 42 mmol/pH unit per kg muscle, quite comparable to the range of 39–77 mmol/pH unit per kg muscle found with mammalian muscle by similar methods (Heisler and Piiper, 1971) or pH-sensitive glass microelectrodes (Aickin and Thomas, 1977; Ellis and Thomas, 1976). These very high buffer values help to explain why extracellular pH changes had so little effect on pH_i. The buffer value may be referred to intracellular water by assuming that all the buffer is intracellular and that 1 kg muscle has 0.67 kg cell H_2O, 0.12 kg extracellular H_2O, and 0.21 kg dry material (Desmedt, 1953; Adrian, 1960). With this correction, our buffer value becomes 63 mmol/pH unit per kg cell H_2O.

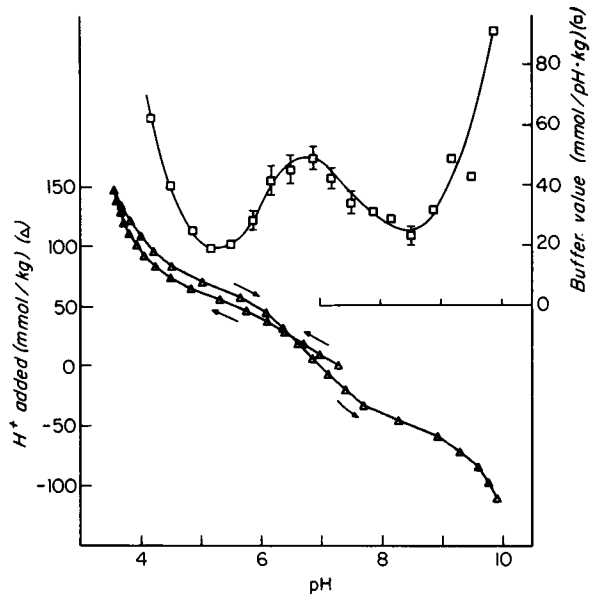


FIGURE 10 Titration curve and calculated buffer values of muscle homogenates. The titration (triangles) was done first by additions of HCl and then back titration with NaOH at 0°C with strong stirring in air. Arrows indicate the direction of titration. Buffer values (squares) are mean values calculated from the slopes of three separate titration curves and are given in millimoles per pH unit per kilogram muscle. Buffer values should be multiplied by 1.5 to get millimoles per pH unit per kilogram cell water. Vertical bars give SEM of buffer values where more than two values were determined.

Knowing the myoplasmic buffer properties as a function of pH, we predicted the changes of myoplasmic pH after cutting the ends of the fiber in the buffered solutions of Table I. The myoplasmic buffer was assumed to be nondiffusible and the applied buffer was assumed to diffuse down the axis cylinder from both ends. At every stage in the diffusion, the appropriate buffer reactions were assumed to be at equilibrium. The length of the fiber was divided into 40 equal compartments for the calculation, and the diffusion equation was integrated in time steps Δt of 0.25 s by the Euler method. For a substance of concentration C and diffusion coefficient D, the change of concentration ΔC in one time step is

$$\Delta C_m = (C_{m+1} - 2C_m + C_{m-1})D\Delta t/(L/40)^2 \qquad \text{(A1)}$$

where the index m refers to the mth compartment. In each time step, Eq. A1 was solved for the diffusion of the basic form B and the acidic form BH^+ of the applied diffusing buffer, and then B_m was incremented by $\Delta B_m + \Delta H_m^+$, and BH_m^+ was incremented by $\Delta BH_m^+ - \Delta H_m^+$, where ΔH_m^+ represents protons transferred from the diffusing buffer to the fixed buffer of the muscle interior in one time step. For the first time step when mobile buffer reaches a compartment, ΔH_m^+ is given implicitly by

$$\Delta H_m^+/\beta = pK - pH_m + \log_{10}[(\Delta B_m + \Delta H_m^+)/(\Delta BH_m^+ - \Delta H_m^+)] \qquad \text{(A2)}$$

where pK is the pK_a of the diffusing buffer and β is the pH-dependent buffer value of myoplasm taken from Fig. 10 but multiplied by 1.5 to be referred to fiber water. From then on, ΔH_m^+

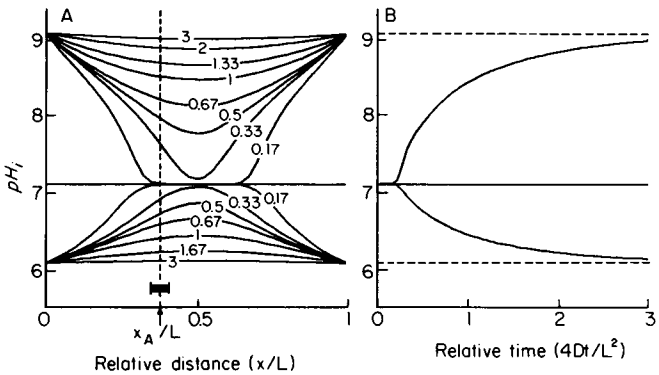


FIGURE 11 Simulation of buffer diffusion through the myoplasm of muscle fibers using the equations given in Appendix. In the calculation, 100 mM buffer solutions at pH = 6.15 or pH = 9.17 were assumed to be applied to the cut ends of a muscle fiber whose initial internal pH is 7.15. Times and lengths are normalized to be dimensionless numbers. For our experiments, the fiber length L is 1,330 μm, the buffer diffusion coefficient D is 0.25×10^{-5} cm^2/s, and the center X_A of the clamped membrane is 500 μm from the end in the current-delivering pool. Under these conditions, the time 1.0 corresponds to 30 min. (A) Calculated pH_i along the fiber after different times after applying buffer to the cut ends. The vertical broken line and horizontal bar indicate the location of the clamped membrane (X_A); (B) Time-course of pH changes at that point.

is given by

$$\Delta H_m^+/\beta = \log_{10}\left(\frac{B_m + \Delta B_m + \Delta H_m^+}{BH_m^+ + \Delta BH_m^+ - \Delta H_m^+} \cdot \frac{BH_m^+}{B_m}\right) \qquad (A3)$$

To cast Eqs. A2 and A3 in usable, explicit form, the antilog was taken of both sides and the left-hand sides expanded as Taylor series. The computer program was tested by comparing the results of several simple limiting cases with analytical solutions. The time for $B + BH^+$ in the center of the fiber to reach 50% of the value of the cut ends was 812 s with the parameter values given below.

Fig. 11A shows the predicted myoplasmic pH vs. position in a fiber at various times after exposing both ends to a 100 mM pH 9.17 tricine or pH 6.15 MES buffer solution. The pK_a's of MES and tricine at 12°C were taken as 6.24 and 8.82 (Good et al., 1966) and the starting pH_i of the fiber as 7.14. The vertical dashed line marks the position of the segment of fiber under voltage clamp, and Fig. 11B shows the time-course of pH_i at that position. The time scale has been left dimensionless to permit use of the figure for various values of D and L. Space-filling molecular models show MES and tricine molecules to be about the same size as the tetraethylammonium ion (TEA). From the limiting equivalent conductivity of TEA at 12°C (Robinson and Stokes, 1965), the diffusion coefficient is 0.61×10^{-5} cm^2 s^{-1}, but we used a value of 0.25×10^{-5} cm^2 s^{-1} for D of the buffers, as Kushmerick and Podolsky (1969) showed that the diffusion of small molecules in myoplasm is about half as fast as in free solution. With this value D and a fiber length L of 1,330 μm, a dimensionless time of 1.0 in Fig. 10 corresponds to 30 min. In that time, the predicted change of pH_i is only two-thirds complete.

One direct experimental check was made on the calculations. A whole muscle was cut into 2-mm slices, which were soaked for 60 min in the 100 mM MES or tricine buffers at 12°C. The same experiment was simulated numerically. Then the slices were homogenized and their mean pH determined to be 6.2 and 8.3, compared with predicted values of 6.2 and 8.6.

We initially underestimated the difficulty of changing pH_i and in many experiments had cut the ends of fibers in solutions with 40 mM phosphate buffer at pH 9 (I9P) or with 57 mM phosphate buffer at pH 6 (I6P) to change pH_i. For example, the fibers used for Figs. 1 and 3 were cut in phosphate buter at pH 9. However, both because of the lower buffer concentration and because of less favorable pK_a's, the above type of calculation predicts that these buffers change pH_i merely from 7.1 to 7.3 (cut in solution I9P) or to 6.8 (cut in solution I6P) after 30 min, so these maneuvers were relatively ineffectual. These experiments with internal phosphate buffer were reported in an abstract (Schwarz et al., 1977) with an over-optimistic estimate of the resulting change of pH_i. The values of pH_i given there should be replaced by 7.3 and 6.8.

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