pH-dependent binding of local anesthetics in single batrachotoxin-activated Na^+ channels

Cocaine vs. quaternary compounds

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ABSTRACT The effects of internal and external pH on the binding kinetics of local anesthetics (LAs) were studied in single batrachotoxin-activated Na^+ channels incorporated into planar bilayers. With internal quaternary QX-314 and RAC421-II drugs, the binding interactions were little affected by either external or internal pH. With tertiary cocaine, the binding kinetics were drastically altered by pH. A decrease in the internal pH from 9.3 to 6.2 decreased the apparent equilibrium dissociation constant (K_d) of internal cocaine by more than 100-fold. This increase in the binding affinity was mostly accounted for by an increase in the apparent cocaine on-rate constant (k_{on}) of ~80-fold. The cocaine off-rate constant (k_{off}) was little changed (between 3–4 s^{-1}). These results demonstrate quantitatively that the charged form of cocaine is the active form for BTX-activated Na^+ channels. Surprisingly, the apparent pK_a of cocaine near its binding site was estimated to be 1.4 units lower than that in bulk solution (7.1 vs. 8.5), indicating that the LA drug encounters a relatively hydrophobic environment. Opposite to the internal pH effect, a decrease of external pH from 8.4 to 6.2 increased the K_d value of internally and externally applied cocaine by ~8- and ~25-fold, respectively. External pH effect was primarily mediated by modulation of k_{on}; k_{off} was again relatively unaffected. Our findings support a model in which neutral cocaine can readily cross the membrane barrier, but needs to be protonated internally to bind to its binding site.

INTRODUCTION

Local anesthetics block voltage-gated Na^+ channels of nerve, muscle, and cardiac tissues in a very complicated manner that may involve both normal Na^+ channel activation and inactivation processes (for review, see Hille, 1984). However, the complicated Na^+ channel gating mechanism can be altered by batrachotoxin (BTX), which is known to eliminate the Na^+ channel inactivation and shift the activation process by >50 mV toward the hyperpolarizing direction (Khodorov, 1978). As a result, the BTX-modified Na^+ channel remains open for >95% of the time at voltages more positive than −60 mV. By using BTX and a bilayer system, the detailed interactions between the local anesthetic and the open Na^+ channel can be directly studied at voltages ranging from −50 to +50 mV at the single-channel level without the complications of the gating processes (Moczydlowski et al., 1986; Wang, 1988).

Previous studies on LA-induced closures in BTX-activated Na^+ channels have revealed certain binding characteristics of local anesthetics in the bilayer system. First of all, different LA drugs appear to induce very different mean closure times in bilayers, ranging from <10 ms to longer than several seconds. For example, quaternary QX-314 induces short-lived closures of <10 ms, too short to be resolved in this system. In contrast, (−)cocaine, the naturally occurring alkaloid found in the *Erythroxylon coca* leaves and the first clinically used local anesthetic (for history, see Ritchie and Greene, 1985), induces long-lasting closures on the order of 300 ms. Also, the binding site of cocaine is found highly stereoselective because synthetic (+)cocaine fails to induce discrete channel closures even at a concentration of 4 mM (Wang, 1989), thus demonstrating that a specific receptor site is present in the BTX-modified Na^+ channel for LA drugs.

After these initial studies of LA action in the simple bilayer system, we have now investigated the pH effects on LA binding in single BTX-activated Na^+ channels. We have restricted our experiments to cocaine and two quaternary amine LAs, QX-314 and RAC421-II, as controls. Cocaine, being a tertiary amine LA, has a pK_a value of 8.5 in aqueous solution (Katzung, 1982). Therefore, in an aqueous solution of pH 8.5, 50% of cocaine molecules are in their neutral form. Unlike cocaine, QX-314 and RAC421-II bear a permanent charge and cannot pass through the lipophilic bilayer. In bilayers, QX-314 and RAC421-II are active only when applied internally, because these drugs cannot enter the Na^+ channel through its narrow external mouth (Hille, 1984).

Numerous studies on the pH dependence of tertiary amine LA block of macroscopic Na^+ currents have been

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performed in intact tissues (for reviews, see Narahashi, 1971; Hille, 1984). Narahashi (1971) surveyed the pH results in the literature and proposed the following LA-Na^+ channel interactions: (*a*) the active form of the tertiary LA drugs is the charged form; (*b*) only the neutral form can easily penetrate the cell membrane; and (*c*) the externally applied tertiary LA drugs must first pass through the lipid bilayer and subsequently become protonated intracellularly to be effective. Supports for these conclusions have continued to grow recently (see Hille, 1984) but with some modifications. For example, the neutral form of tertiary LA drugs such as lidocaine may also bind to the Na^+ channels but with a fast lipophilic excape route (Schwarz et al., 1977). External H^+ ions are believed capable of protonating the bound neutral LA drugs and thereby preventing their escape via the lipophilic pathway. Surprisingly, intracellular pH has little effect on external lidocaine in skeletal muscle fibers (Schwarz et al., 1977). In general, changes in pH have no effect on quaternary LA action suggesting that LA binding site is not directly affected by the H^+ ions.

Recognizing the physiological importance of pH effects on LA action, we have reexamined Narahashi's three hypotheses at the single channel level. Although the bilayer experiments require the presence of BTX, our results are in excellent agreement with his theory. However, we were unable to observe direct evidence that the neutral form of cocaine can bind favorably with the BTX-activated Na^+ channels. Furthermore, internal pH has profound effects on the action of external tertiary amine LAs, a phenomenon that is different from the results obtained in muscle fibers. Preliminary results of this study have been reported in an abstract form (Nettleton and Wang, 1989).

MATERIALS AND METHODS

Chemicals

Synthetic phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were purchased from Avanti Polar Lipids (Birmingham, AL). BTX was a generous gift of Dr. John Daly, Laboratory of Bio-organic Chemistry, National Institutes of Health, Bethesda, MD. Cocaine · HCl was purchased from Mallinckrodt, Inc., St. Louis, Mo. QX-314 · Cl and RAC 421-II · Cl were supplied by Dr. Bertil Takman of Astra Pharmaceutical Products, Inc., Worcester, MA. Tetrodotoxin was obtained from Calbiochem-Behring, Corp., San Diego, CA. All other chemicals were reagent grade from commercial sources used without further purification. Cocaine-HCl, QX-314, and RAC 421-II were each dissolved in standard aqueous solution (200 mm NaCl, 0.2 mM EGTA, 10 mM Hepes-NaOH, pH 7.4) at 100–200 mM stock concentration. Stock cocaine solution was aliquoted, and stored in −70°C until use. Stock solutions of QX-314 and RAC421-II were stored in −20°C. All experiments were performed at a room temperature of 23 ± 2°C.

Membrane preparation

Plasma membrane vesicles were prepared from rabbit skeletal muscle as described by Moczydlowski and Latorre (1983) and Moczydlowski et al. (1984). Light vesicles banding on a cushion of 30% sucrose (wt/vol) were pelleted, resuspended at ~10 mg protein/ml in 300 mM sucrose and stored at −70°C. The membrane preparation remains suitable for bilayer studies for >1 yr.

Planar bilayers and Na channel insertion

Planar bilayers were cast on 100–200 μm holes in PVC partitions from decane solutions containing PE (13.4 mg/ml) and PC (6.7 mg/ml). Ionic currents were monitored at constant holding voltage using a List EPC-7 voltage clamp (Medical Systems Corp. Great Neck, NY). In general, plasma membrane vesicles (~10 μg/ml) were added to the *cis* side of the bilayer and the voltages were alternated from −65 to 65 mV every 10 s to facilitate the incorporation. Insertion of Na^+ channels

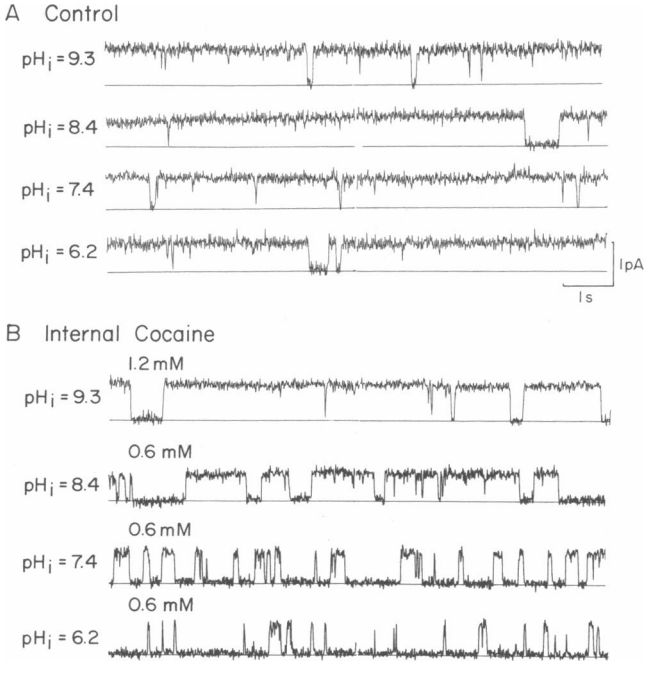

FIGURE 1 Internal pH affects cocaine potency in BTX-activated Na^+ channels. Current traces of single Na^+ channels at 0 mV are shown from several bilayers with pH_e held constant at 7.4. (*A*) Control current records at various pH_i conditions without cocaine present. In the range shown, pH has little effect on the Na^+ channel alone. Records with intrinsic closing events were selected for baseline determination. (*B*) Current traces in the presence of cocaine at various pH_i. The fractional closed time is reduced from 87% at pH_i = 6.2 to 11% to at pH_i = 9.3. The cocaine-induced closures were infrequent at pH 9.3 so that it was necessary to double the usual cocaine concentration to observe enough closing events for analysis. Solid lines in each current trace indicate the zero current level. Inward currents are displayed upward for comparison. All bilayers were formed under asymmetrical 300 mM external and 50 mM internal NaCl conditions.

could be detected in the presence of 100 nM BTX added from the *cis* side of the bilayer, essentially as described by Krueger et al. (1983) and Green et al. (1987). Under asymmetrical 300 mM external and 50 mM internal NaCl conditions single BTX-activated Na^+ channels gave rise to about −0.7 pA current amplitude deflection at $V = 0$ mV. Inward current traces were displayed upward in the figure for comparison. These Na^+ currents were blocked by TTX in a voltage-dependent manner and were activated around −100 mV. All voltages are defined as intracellular voltage and the external face of Na^+ channels is defined as zero voltage.

Currents were filtered at 50–100 Hz, recorded at 100 Hz, stored, and later analyzed by an AT computer as described (Wang, 1988). Bilayers containing one channel could be easily analyzed by pClamp software (Axon Instruments, Inc., Burlingame, CA) to find k_{on}, k_{off}, and k_d from the mean open and closed times. In some experiments bilayers containing two channels were analyzed to find the fractional open time (f_o). K_d could then be estimated by the following equation:

$$1 - f_o = [LA]/([LA] + K_d), \quad (1)$$

where [LA] is the local anesthetic concentration. Bilayer records with more than two channels were discarded. Simulations of single channel events were performed by CSIM software (Axon Instruments, Inc.) and the computer-generated data were then analyzed by the same pClamp program.

Buffers for various pH conditions

Unless otherwise stated, the initial *cis* solution contained 300 mM NaCl, 0.2 mM EGTA, 10 mM Hepes-NaOH, pH 7.4, and the *trans* solution contained 50 mM NaCl, 0.2 mM EGTA, 10 mM Hepes-NaOH, pH 7.4. After control records were taken, the pH at one side was then either raised or lowered by the addition of appropriate buffers. The addition of 6 μl 1 M MES acid ($pK_a = 6.15$) per 1 ml standard solution lowered the pH to 6.2 ± 0.2. The addition of 10 μl 1 M Tris base pK_a ($pK_a = 8.3$) per 1 ml standard solution raised the pH to 8.3 ± 0.1. pH was raised to ~9.2 with ~10 μl 0.5 M CAPS NaOH base ($pK_a = 10.4$) per 1 ml standard solution. Under this pH range (6.2–9.3) drug-free Na^+ channels remained open most of time (≥95%). The final pH value in the aqueous solution was always measured at the end of experiments.

RESULTS

Internal pH affects the potency of internally applied cocaine

The extent of protonation of tertiary amine drugs in bulk solution is determined by the pH. Lowering the pH favors the protonation and hence the charged form of the drugs.

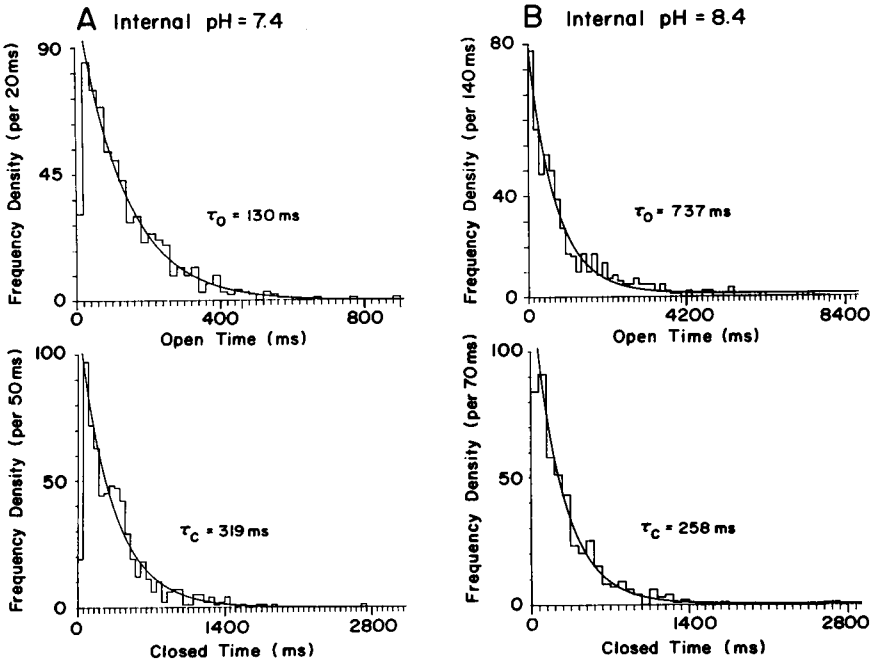

FIGURE 2 Histograms of open and closed time distribution in the presence of cocaine. (*A*) At pH 7.4, both the open time (*top*) and the closed time (*bottom*) distributions at 0 mV can be fitted to a single exponential with a time constant of τ_o and τ_c, respectively. Closures of <30 ms were not counted to exclude the intrinsic closing events. Cocaine was applied internally at 600 μM. The upper panel curve was drawn according to the following term: $N(t) = N \cdot \exp(-t/\tau_o)$ where $N(t)$ is the number of open events per 29 ms bin, N is the number of events in the population $t = 0$, and τ_o is the mean open time; $N = 109$, $\tau_o = 130$ ms. The same term was used for the lower panel where $N = 114$, $\tau_c = 319$ ms. In seven separate experiments τ_o and τ_c values were 174 ± 42, and 312 ± 57, respectively. (*B*) At pH 8.4, the τ_o was about sixfold longer than the τ_o at pH 7.4 (*top*) whereas the τ_c was little affected (*bottom*). Cocaine was applied internally at 600 μM. $N = 79$, $\tau_o = 737$ ms for upper panel and $N = 467$, $\tau_c = 258$ ms for lower panel. In three separate experiments at pH 8.2–8.4, τ_o and τ_c values were 1,045 ± 348 ms and 309 ± 47 ms, respectively. All bilayers were formed under 300 mM external and 50 mM internal NaCl conditions.

To examine which forms of LA drug in solution interact with the Na^+ channels, we performed a series of experiments in planar bilayers at various levels of internal pH (pH_i). Fig. 1 shows typical Na^+ current traces recorded at four different levels of pH_i with and without internally applied cocaine. With a given bilayer, changes in pH_i alone from pH 6 to 9 did not have significant effects on Na^+ current amplitude and kinetics at $V = 0$ mV. The amplitude of Na^+ currents in different bilayers, however, varied somewhat with a standard deviation of 0.68 ± 0.05 pA ($n = 23$). Below pH 6.0, Na^+ current amplitude is greatly reduced and open channel noise is increased as if the currents are blocked by the internal H^+ ions (Woodhull, 1973). Because of this intrinsic block by H^+ ions we excluded studies on the pH_i below 6.

When cocaine is applied internally, a change in pH_i can greatly alter its potency. At pH 7.4, cocaine induces Na^+ channel closures (Fig. 1) with a fractional closed time (f_c or $1 - f_o$, see Eq. 1) of 72% in a 5-min record at 600 μM, corresponding to a K_d value of 233 μM. At pH 6.2, the f_c increases to 87% ($K_d = 90$ μM), whereas at pH 8.4, f_c decreases to 21% ($K_d = 2.3$ mM). At pH 9.3 and with 1.2 mM cocaine f_c is further reduced to 11% ($K_d = 9.7$ mM). These results demonstrate that lowering the pH_i increases the cocaine potency in inducing channel closures and suggest that the charged form of cocaine interacts with the BTX-activated Na^+ channel directly.

On-rate constant of cocaine binding is strongly pH-dependent

The current traces in Fig. 1 indicate that the open times are strongly dependent on pH_i while the closed times are not. The kinetics of the Na^+ channel–cocaine interactions under these conditions can be directly characterized by measuring the open and closed time distributions (Fig. 2). Both the open and closed time histograms can be fitted to a single exponential with a time constant of τ_o and τ_c, respectively. At pH 8.4 (Fig. 2 *B*), the τ_o and τ_c values are 737 and 258 ms, respectively, whereas at pH 7.4 (Fig. 2 *A*) they are 130 ms and 319 ms, respectively. Fig. 3 shows that the τ_c values are relatively constant at different pH_i but the τ_o values increase nonlinearly from pH 6.2 to 9.3.

We have found previously that τ_o is drug concentration–dependent but τ_c is not (Wang, 1988). Assuming a general binding scheme of:

$$L + O \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} L \cdot O$$

Scheme A

where L is the drug, O is the open channel and L · O is the bound and closed channel. The τ_o and τ_c time constants can be related to the on-rate (k_{on}), off-rate (k_{off}), and the

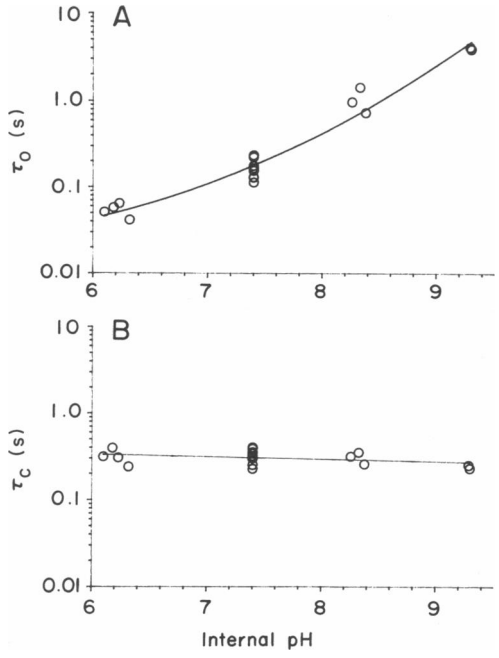

FIGURE 3 Effects of internal pH on open and closed time constants. The τ_o (*A*) and τ_c (*B*) values were measured at 0 mV and plotted against pH_i. Panel *A* shows that an increase of pH increases the τ_o continuously in a nonlinear fashion. Unlike open time, the closed time, τ_c is relatively unaffected by pH_i (*B*). All time constants were measured in the presence of 600 μM internal cocaine except for pH_i 9.3 where 1.2 mM cocaine was applied. The τ_o value at pH_i 9.3 was doubled to compensate for the difference in cocaine concentration. For each time constant, more than 300 events were measured and analyzed as described in Fig. 2. Bilayers were formed under 300 mM external and 50 mM internal NaCl conditions. External pH was kept constant at pH 7.4.

equilibrium dissociation (K_d) constants by the following equations:

$$k_{on} = 1/(\tau_o[L]) \quad (2)$$

$$k_{off} = 1/\tau_c \quad (3)$$

$$K_d = k_{off}/k_{on} \quad (4)$$

where [L] is the drug concentration. We calculated k_{on}, k_{off}, and K_d, using the applied drug concentration for [L] and plotted the K_d values versus pH_i (Fig. 4). It is noteworthy here that different concentration of BTX (from 50 to 250 nM) do not affect the kinetics of the cocaine-induced block. Consequently, cocaine-induced block is not due to the dissociation of BTX from its binding site while cocaine is bound. Spontaneous disappearance of Na^+ currents does occur during our experiments but happens infrequently as described previously by Green et al. (1987).

The rather specific effect of pH_i on τ_o is consistent with the hypothesis that the charged form of cocaine interacts

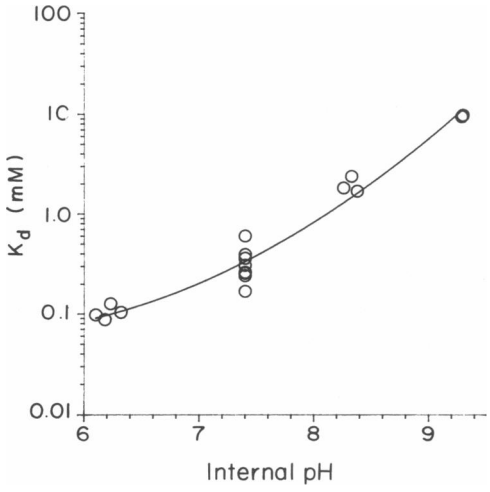

FIGURE 4 K_d values of cocaine at various internal pH. K_d values were determined according to Eqs. 2–4 and plotted against pH_i. The results show that the lower the internal pH the lower the K_d value. The data was fitted to a second order linear regression but the curve has no theoretical significance. The external pH was kept constant at 7.4. Bilayers were formed under 300 mM external and 50 mM internal NaCl conditions. Kinetic data were analyzed at 0 mV membrane potential.

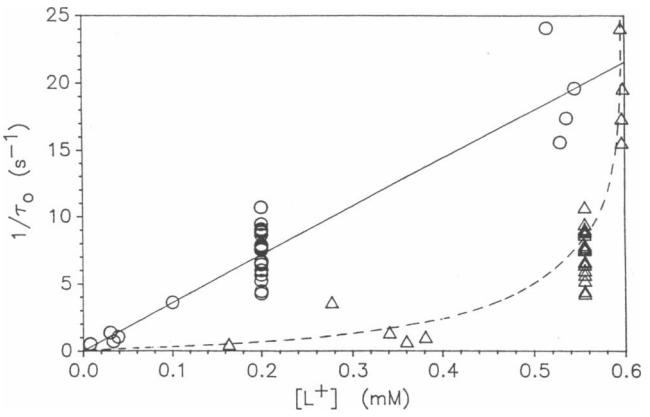

FIGURE 5 Estimate of true k_{on} according to the charged form of cocaine concentration. The $1/\tau_o$ vs. $[L^+]$ plot shows a nonlinear relationship for a chosen pK_a of 8.5 (△, *dashed line*), the pK_a of cocaine in bulk solution, and a linear relationship for a pK_a value of 7.1 (o, *solid line*), the estimated pK_a near the binding site. The solid line represents the least square fit of the data (correlation coefficient, $r = 0.94$) whereas the dashed line is fitted by eye. The slope of the solid line yields the true k_{on} for the charged cocaine molecule at $\sim 3.6 \times 10^4$ M^{-1} s^{-1}. The k_{on} value is calculated as $1/(\tau_o\ [L^+])$ where $[L^+] = [L]_T \cdot [H^+]/(K_a + [H^+])$ and $[L]_T$ is the applied total cocaine concentration. This equation for estimating $[L^+]$ is derived directly from the Henderson-Hasselbach equation. All bilayers were formed under 300 mM external and 50 mM internal NaCl conditions and currents were recorded at 0 mV.

directly with the BTX-activated Na^+ channel. Because cocaine has a pK_a of 8.5, the concentration of the charged active form should change according to the pH. Consequently, the measured τ_o and k_{on} would vary with pH_i. Attempts were therefore made to estimate a true k_{on} for the charged cocaine binding.

Estimate of the true on-rate binding and the pK_a of cocaine in the channel

From Eq. 2, we would expect $1/\tau_o$ to be linearly related to the drug concentration [L] with a slope equal to the true k_{on}. Three assumptions were made to estimate this true k_{on}: (*a*) that the concentration [L] would be equal to charged cocaine concentration; (*b*) that pH and H^+ activity in the channel are the same as in the internal solution because of channel's large inner vestibule (Hille, 1984); (*c*) that the ratio of the charged to the neutral form follows the Henderson-Hasselbach equation

$$pH = pK_a + \log([A]/[HA]), \qquad (5)$$

where [A] is neutral species, [HA] is charged species, and [A] + [HA] equals the applied cocaine concentration.

Using Eq. 5, we calculated the charged form concentration, [HA], by choosing various pK_a values for cocaine. The calculated [HA] was then plotted vs. $1/\tau_o$ to obtain k_{on}. Fig. 5 shows that with a pK_a value of 8.5, the $1/\tau_o$ values plotted against [HA] (Fig. 5, *dashed line*) give a nonlinear relationship. This result contradicts with the assumption that the pK_a of cocaine near the LA binding site is the same as in the bulk solution. If we use the pK_a value of 7.1, on the other hand, a linear relationship ($r = 0.94$) can be obtained which yields an on-rate constant of 3.7×10^4 M^{-1} s^{-1} (Fig. 5, *solid line*). This pK_a is the best fit of all other possible pK_a values. We suggest, therefore, that the charged cocaine molecule encounters a very different environment within the channel as compared to that in bulk solution. The lower pK_a value also indicates that near the cocaine binding site the milieu is relatively hydrophobic and the charged cocaine molecule tends to lose its proton.

External pH affects the internal cocaine potency

It is known that external Na^+ ions can influence the binding interactions between LA drug and Na^+ channels (Cahalan and Almers, 1979; Wang, 1988). To see whether external H^+ ions can also affect such interactions we studied the cocaine binding rates at various external pH (pH_e) while keeping the internal pH at 7.4. Typical current traces from these experiments are shown in Fig. 6. In contrast to the pH_i experiments we found that raising the pH_e increases the cocaine potency.

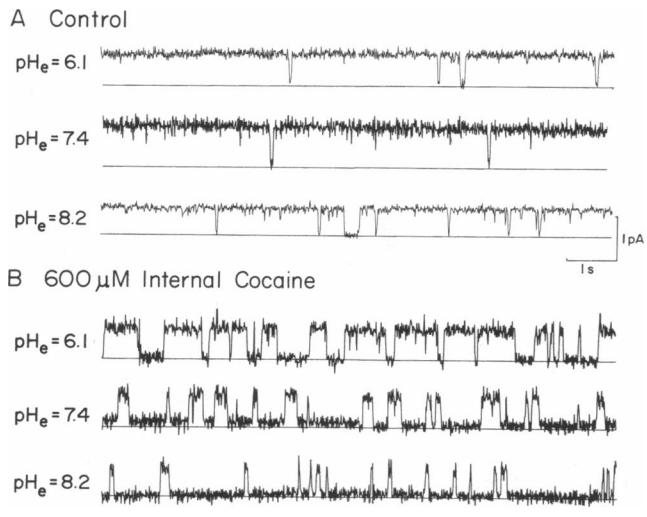

FIGURE 6 External pH affects the cocaine potency. Current traces of single Na channels were from different bilayers at three different external pH. The internal pH remained constant at 7.4 (*A*) Control current records without cocaine present. In this pH range there was no significant effect of pH_e on the channel kinetics. (*B*) Current traces with 600 μM internal cocaine. Contrary to internal pH effects, increasing pH_e enhances internal cocaine potency. At $pH_e = 6.1$, the fractional open time, f_o, was measured to be 54% whereas at $pH_e = 8.2$, f_o equaled 13%. All bilayers were formed under 300 mM external and 50 mM internal NaCl concentration.

The kinetics of the binding interactions were obtained by measuring τ_o and τ_c and the rate constants were calculated by using Eqs. 2 and 3. Fig. 7 shows that the pH_e effect on cocaine potency is due mainly to a change in the on-rate. No changes in voltage-dependent binding interactions were found under various pH_e (Fig. 8). Thus, the voltage dependence of cocaine binding is not a factor in determining the altered τ_o value at various pH. There is about an eightfold difference in K_d at pH 6.2 and 8.4, ranging from 800 μM (at pH 6.2) to 100 μM (at pH 8.3). This result demonstrates that external H^+ ions affect the cocaine binding strongly. Furthermore, because external H^+ ions reduce the on-rate of cocaine binding (Fig. 7 *B*), direct protonation of the internal cocaine molecule by H^+ ions through external mouth of Na^+ channels does not occur significantly in the bilayer. This could be due to neutralization of H^+ ions within the inner vestibule by the internal buffer.

External and internal H^+ ions have little effect on quaternary LA action

One explanation for the pH_e effect on cocaine potency is that H^+ ions from the external side can enter the channels and, like Na^+ ions, antagonize the cocaine binding. To check this possibility we examined the pH effect on the potency of the quarternary LA drugs, QX-314 and

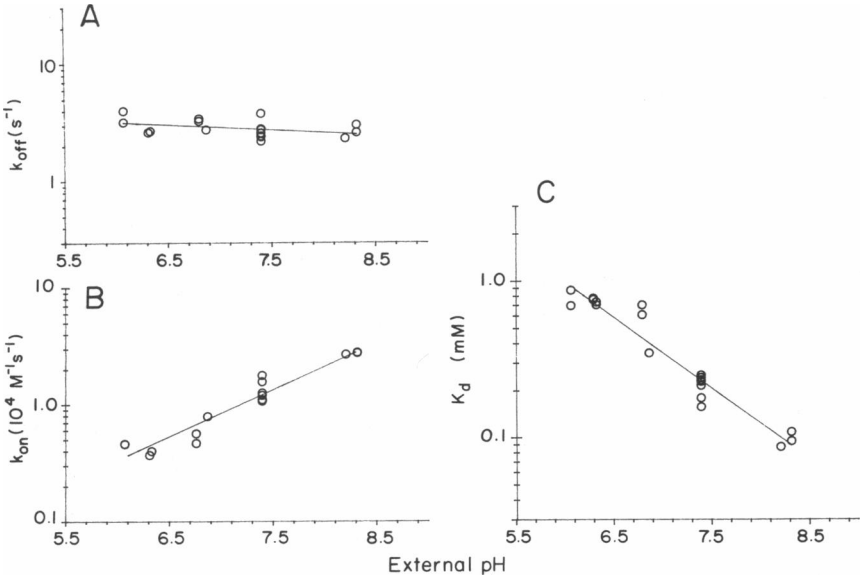

FIGURE 7 Cocaine binding kinetics at various external pH. The k_{off}, k_{on}, and K_d values were plotted against pH_e. (*A*) As with internal pH, k_{off} values appeared insensitive to pH_e, with a slope of only -0.045 (log k_{off})/pH. (*B*) k_{on} values, on the other hand, were quite sensitive to changes in the external pH. Data from five sets of experiments were plotted and the rate of change in log k_{on}/pH was determined for each set. The average rate of change was 0.50 (log k_{on}/pH) with a standard deviation of $\pm$ 0.1. (*C*) The slope for log K_d vs. pH was found to be -0.45 by a first order linear regression. Bilayers were formed under asymmetrical conditions, 300 mM external and 50 mM internal NaCl concentration. Cocaine at 600 μM was applied internally.

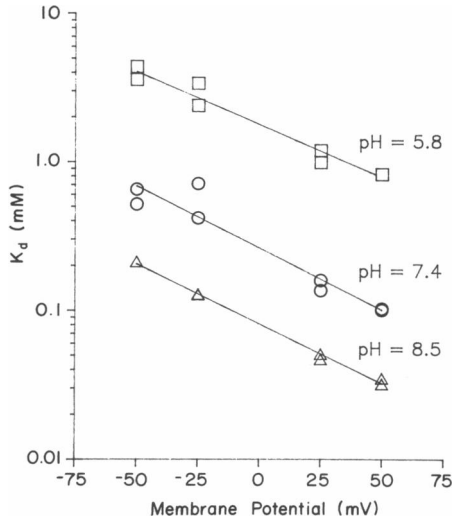

FIGURE 8 Voltage-dependence of K_d values under different external pH. Although external pH affects the K_d values significantly, it does not alter the voltage dependence of cacaine binding. The average slope (log K_d/100 mV) was -0.78 ± 0.07 ($n = 4$). K_d values were estimated according to Eq. 4 at voltages between -50 mV and $+50$ mV from three separate experiments. Bilayers were formed in 200 mM NaCl symmetrical conditions. Cocaine was applied internally at 600 μM.

RAC421-II. We found that neither pH_i nor pH_e had much effect on the potency of QX-314 (data not shown). Similarly, changes in either internal or external pH failed to affect the binding of RAC421-II significantly (Table 1). These results imply that H^+ ions, unlike external Na^+ ions, have little effect on the LA-receptor binding complex. Thus, it seems unlikely that H^+ ions (~1 μM at pH 6) repel the bound LA or elicit some allosteric effects on the LA-receptor complex.

TABLE 1. Open and closed time constants of RAC421-II induced block at various pH conditions

pH_e	pH_i	τ_o	τ_c	No events	K_d
		ms	*ms*		*mM*
7.4	6.3	131	19.2	1615	6.83
7.4	8.4	126	18.3	1036	6.96
6.2	7.4	196	17.5	2062	11.1
7.4	7.4	125	17.3	2831	7.97
8.4	7.4	117	14.6	548	8.00

The time constants τ_o and τ_c for open and closed events, respectively, were obtained by fitting the data as performed in Fig. 2. No cut-off times were applied because the closed events were extremely frequent and very short. pH_e is the external pH and pH_i is the internal pH. The number of events for τ_o and τ_c determinations is listed. Despite poor resolution of our bilayer system, the data show that pH has little effect on RAC421-II–induced closures. All bilayers were formed in 300 mM external and 50 mM internal NaCl conditions. Kinetic data were measured at $V = 0$ mV. K_d was estimated using Eq. 4. A better bilayer resolution at 1 KHz is required to determine accurate kinetic values of RAC421 compound in the future.

Both external and internal pH strongly affect the external cocaine potency

The neutral form of LA drugs is believed to pass through the bilayer membrane easily. To check whether cocaine in its neutral form can penetrate the membrane we examined the pH effects on externally applied cocaine. Consistent with the notion that neutral cocaine can pass through the bilayer, we found that raising pH_e increases the apparent on-rate constant significantly (Fig. 9), whereas the apparent off-rate constant is little changed. Consequently, the calculated apparent K_d decreases significantly. An increase of k_{on} again suggests that a higher concentration of cocaine are available to bind with the channel when pH_e is raised.

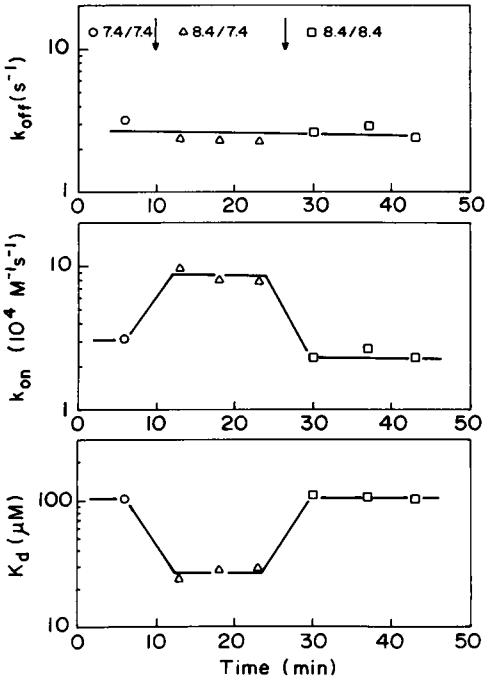

FIGURE 9 Effects of external and internal pH on externally applied cocaine. Data were recorded from the same channel at 0 mV from seven trials each 5 min long. Kinetics were first measured at pH_e/pH_i = 7.4/7.4 (O's), pH_e was then raised from 7.4 to 8.4 (Δ), and last pH_i was raised from 7.4 to 8.4 (□). (*A*) k_{off} is little affected by the pH changes. (*B*) k_{on} and (*C*) K_d of external cocaine are altered by both internal and external pH changes. Raising pH_e increases the external cocaine potency whereas raising pH_i gives rise to an opposite effect. This potency change is mainly due to a change in the k_{on} value. Similar results were obtained in three separate experiments at pH ranging from 6.2 to 8.4. The bilayer was formed at 300 mM external and 50 mM internal NaCl conditions.

Schwarz et at. (1977) reported that pH_i has little effect on external lidocaine, presumably because the activation gate is closed most of the time in intact tissue, limiting the access of internal H^+ ions to the LA binding site. This hypothesis can be directly tested in BTX-activated Na^+ channels at voltages larger than -60 mV. Under this condition, we predict that pH_i will have profound effects on external cocaine, because access of H^+ ions to the channel pathway is no longer limited by the gate. Fig. 9 shows that an increase in pH_i reduces the external cocaine potency significantly. This pH effect is similar to that found for internal cocaine, again suggesting that the charged form of cocaine is the active form at the internal side of Na^+ channels. Also, consistent with this notion, we found that a decrease in pH_i increases the external cocaine potency (data not shown). Together, our result suggests that neutral cocaine penetrates the membrane and becomes charged in the pore and in the internal surface of Na^+ channels. The charged cocaine then binds with the Na^+ channels and induces closures (Fig. 9).

From Fig. 9, it is apparent that pH_e could exhibit strong effects on cocaine-Na^+ channel interactions. Initially, it will determine the ratio of charged over neutral form of externally applied cocaine. The concentration of neutral cocaine in the external solution can be derived as follows:

$$[L^\circ]_e = \frac{[L_T]_e}{1 + 10^{pK_a - pH_e}}, \quad (6)$$

where $[L^\circ]_e$ is the concentration of neutral cocaine in external solution, $[L_T]_e$ is the applied cocaine concentration in external solution, pK_a of cocaine in solution is 8.5, and pH_e is the external pH. Because only the neutral cocaine form can pass through the lipid bilayer, the more the external neutral cocaine concentration the higher the neutral cocaine concentration in the pore and in the internal surface of the Na^+ channel,

$$[L^\circ]_i \alpha [L^\circ]_e. \quad (7)$$

Consequently, the effective concentration of charged cocaine in the channel, $[L^+]$, may be indirectly related to the external cocaine concentration as follows:

$$[L^+]_i = [L^\circ]_i 10^{pK_a' - pH_i} \quad (8)$$

$$[L^+]_i \alpha \frac{[L_T]_e \cdot 10^{pK_a' - pH_i}}{1 + 10^{pK_a - pH_e}}, \quad (9)$$

where pK_a' is 7.1 as determined in Fig. 5. Eq. 9 was derived from Eqs. 6–8.

An initial attempt to verify Eq. 9 suggests that $[L^+]_i$ may be drastically modulated by the external pH when the drug is applied externally. Fig. 10 shows that the apparent K_d value for external cocaine is reduced by raising the external pH. A steeper slope of -0.68 was estimated in the log K_d/pH_e plot than that shown in Fig. 7 *C*, which yields a slope of -0.45 for internal cocaine. As a result, the magnitude of this potency change is about 25-fold from $pH_e = 6.2$ to $pH_e = 8.4$, as compared to an eightfold difference for internal cocaine. Because the permeability of neutral cocaine in bilayer membranes is unknown, the exact $[L^+]$ near the Na^+ channel pore cannot be ascertained until this information becomes available. Nevertheless, our results from Figs. 7 and 10 together suggest that pH_e can influence the cocaine potency regardless of the side of cocaine application.

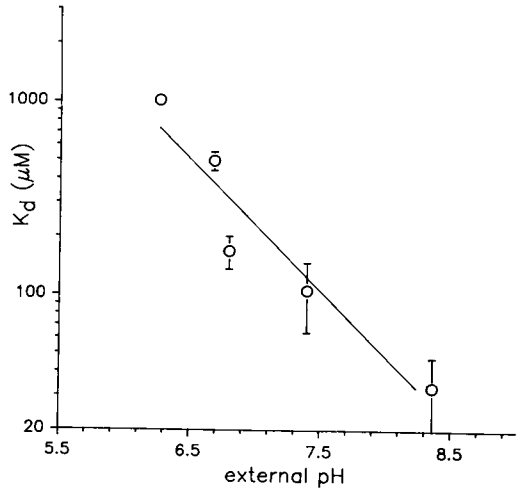

FIGURE 10 Potency of external cocaine at various external pH. The values of K_d were obtained as described in Figs. 4 and 7. Cocaine was applied externally at either 300 or 600 μM. The slope of log K_d/pH is found to be -0.68, which is steeper than the pH_e dependence of internally applied cocaine (Fig. 7 *C*). Bilayers were formed under asymmetrical conditions with 300 mM external and 50 mM internal NaCl concentration.

DISCUSSION

The action of tertiary amine cocaine on BTX-activated Na^+ channels is strongly influenced by the internal as well as external pH conditions. In this report we have measured for the first time these pH effects on cocaine action at the single Na^+ channel level in the bilayer system. In the presence of BTX, detailed kinetic analyses of the (−)cocaine binding are feasible. These analyses reveal the molecular events of (−)cocaine-Na^+ channel binding interactions which occur after the pH changes. Whereas many of our results support previous conclusions based on action potential and macroscopic current records, some interesting differences are observed in the bilayer system. The discrepancy is most likely due to the lack of Na^+

channel gating at voltages more positive than −60 mV in our bilayer studies and will be detailed later.

pH has little effect on quaternary local anesthetics

Before we discuss the action of cocaine under various pH conditions, it is appropriate to distinguish the pH effects on the LA drugs from those on the LA receptor. For quaternary LA drugs, the direct pH effects on LA protonation cannot occur because they contain a permanent charge on the amine group. Hence, any pH effects on these LA-receptor interactions must be due to changes in the receptor site. Furthermore, because quaternary and tertiary amine LA drugs share a common receptor in bilayers (Wang, 1988), quaternary LA can be used as a probe for this purpose. Our results show that pH_i has little effect on either the action of QX-314 or RAC421-II. The K_d values for both quaternary amine drugs remain about the same at pH_i from 6.2 to 8.4. Nor does pH_e alter the K_d values drastically. A ~40% decrease of the τ_o value is observed for RAC421-II at pH_e from 6.2 to 8.4 (Table 1), suggesting that the receptor environment is somewhat modulated by the pH_e. However, this modulation is too small to explain an eightfold (or a 25-fold) difference in the potency of internal (or external) tertiary amine cocaine in this pH range. Our results are, therefore, in agreement with previous reports in normal Na^+ channels which also show that pH has little effect on the LA binding site (Frazier et al., 1970; Schwarz et at., 1977). We conclude that direct effects of pH on the LA binding site is minimal in BTX-activated Na^+ channels.

Active form of (−)cocaine and its site of action

Consistent with the results of tertiary amine LA effects on squid giant axons (Narahashi et al., 1970), our data demonstrate quantitatively that the active form of cocaine for BTX-activated Na^+ channels is the protonated (−)cocaine molecule. We define the active form in this report as the LA form which can readily bind to the open Na^+ channel. Lowering pH_i decreases primarily the open time duration with little effect on the closed time duration. At 600 μM internal cocaine the τ_o value is reduced to ~80-fold when pH_i is lowered from 9.3 to 6.3 (Fig. 3). Because lowering pH_i increases the charged form of cocaine and because a decrease of the τ_o value indicates an increase of the active cocaine concentration (Wang, 1988), we conclude that the active form of cocaine for BTX-activated Na^+ channels is the charged form.

There is other supporting evidence for this conclusion. For example, the binding interaction of cocaine and its receptor is highly voltage dependent. The higher the voltage applied intracellularly, the higher the cocaine binding affinity observed (i.e., Fig. 8). This phenomenon can be interpreted as the charged form of cocaine being driven from the internal aqueous solution toward its binding site by the applied voltage. The degree of the voltage dependence of cocaine binding further indicates the site of cocaine action is located within the pore about halfway through the membrane electric field gradient (Wang, 1988). Also the cocaine binding site appears to be within the channel pore because QX-314 competes directly with cocaine and because permanently charged LA drugs such as QX-314 and RAC421-II can only reach this binding site when applied intracellularly. Furthermore, external Na^+ ions antagonize the LA's binding as if they encounter each other within the channel (Cahalan and Almers, 1979; Wang, 1988). If indeed the binding site is within the channel pore, the site should be accessible to both the charged and neutral forms of (−)cocaine in the aqueous solution. It should be noted here that our experiments do not allow us to exclude the possibility that the neutral cocaine form can also bind to the receptor but at a much slower rate. Previously, it was found that neutral benzocaine at 0.5 mM has an extremely slow τ_o value (~8 s) at 0 mV in BTX-activated Na^+ channels (Moczydlowski et al., 1986), comparable to the τ_o value (~4 s at 0.6 mM) for cocaine at pH 9.3. Thus, at pH 9.3 some of the closing events might be due to the direct binding of the neutral form of cocaine.

Apparent pK_a of cocaine near its binding site

Attempts to estimate the pK_a of LA drugs near the binding site have been reported on several occasions. Narahashi et al. (1970) assumed that the pK_a of LA drugs near the binding site is the same as the value in bulk solution and found that only the charged form of LA drugs was required to explain their results in squid axons at various pH conditions. Similarly, Schwarz et al. (1977) used the aqueous pK_a value of lidocaine in their model to estimate the neutral and charged form of the drug. More recently, Moorman et al. (1986) and Chernoff (1988) reported that pK_a of drugs near the LA binding site, estimated by the recovery time of LA block, may be different from or the same as the pK_a in bulk solution, depending upon the model selected (see Chernoff, 1988). In this study, we were able to estimate the binding kinetics directly at equilibrium under various pH conditions. Such conditions were not possible in previous studies because of the voltage-dependent inactivation of normal Na^+ channels.

Two uncertainties in estimating the pK_a value in our studies are nevertheless unavoidable. First, the changes of

pH in one side of lipid bilayers may create a pH gradient in the channel pathway. Dependent on the location within the channel pathway, the pH value may therefore vary considerably from one site to another. We assume that this is not the case because the inner vestibule of Na^+ channels is relatively large (Hille, 1984; see Fig. 11). This vestibule space should be reached by the internal buffer, e.g., Hepes (mol wt 238.3), which is smaller than cocaine (mol wt 303.35). Practically, the pH gradient cannot be eliminated by changing the pH_e to equal the pH_i because such changes by themselves affect the cocaine binding in opposing ways (Fig. 9). Second, the cocaine-induced closing events at high pH are assumed to be due to the binding of the charged form of cocaine. This is only a simplification because we have no direct ways of distinguishing the closing events caused by the charged form from those caused by the neutral form. Hence, the estimated value for the pK_a of cocaine near the binding site is only an approximation.

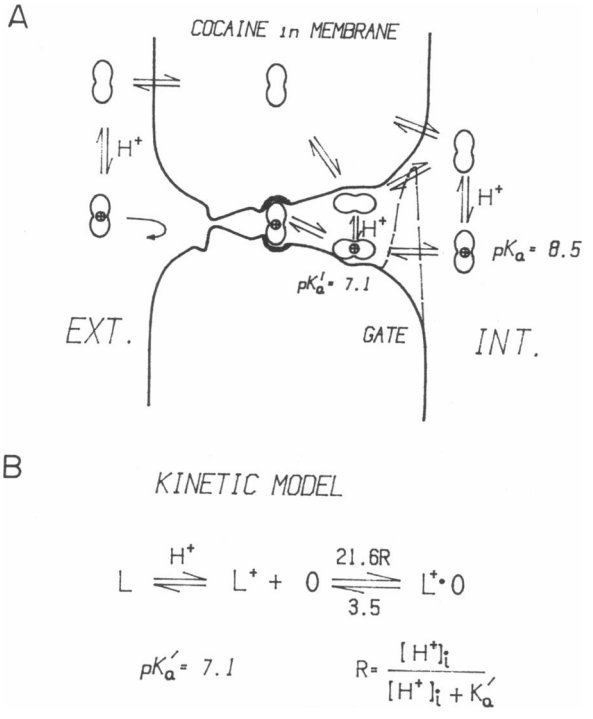

FIGURE 11 Kinetic model for cocaine binding in BTX-activated Na^+ channel. (*A*) Hypothetical view of a local anesthetic binding site within the Na^+ channel conduction pathway. The LA site is relatively hydrophobic, yet only the charged form of LA drugs gains easy access to its binding site. (*B*) Kinetic scheme for cocaine-Na^+ channel interactions. K_a is the acid dissociation constant of the drug near the binding site (for cocaine $K_a = 10^{-7.1}$). Protonation occurs presumably at a diffusion-controlled bimolecular rate constant of $1 \times 10^{10}\ M^{-1}s^{-1}$ for typical amines in solution (Eigen and Hammes, 1963). The deprotonation rate is calculated to be $794\ s^{-1}$ according to the pK_a value. *R* is the ratio of charged forms $[L^+]$ over the total internal drug $[L]_T$ which is determined by the equation: $R = [H^+]_i/(K_a + [H^+]_i)$ This value is thus $[H^+]_i$ dependent. Note that all the rate constants have units of s^{-1}. The external pH indirectly alters the internal $[L]_T$ by controlling the amount of drug diffusing in and out of the external side of the membrane (e.g., Eq. 9).

Our results show that the apparent pK_a of cocaine near its binding site is ~7.1, a value that is significantly lower than the pK_a of cocaine in aqueous solution ($pK_a = 8.5$). Unlike intact tissues, the intracellular and extracellular solutions of our bilayer studies can be directly stirred and their pH can be later measured at the end of experiments. Furthermore, because BTX keeps the activation and inactivation gates open at voltages more positive than −60 mV, the receptor is more accessible to local anesthetics and H^+ ions. In contrast, the normal Na^+ channel only opens very briefly following depolarization, usually for <1 ms under normal conditions (Aldrich et al., 1983). Accordingly, the channel pathway may only be briefly accessible to both local anesthetics and H^+ ions in the intracellular aqueous solution (for detail, see Starmer, 1986). In fact, Schwarz et al. (1977) stated that the pH within the channel may follow the pH_e closely. This phenomenon may render the direct pK_a determination of local anesthetics near the binding site difficult in the normal Na^+ channel. Fig. 11 *B* shows the proposed kinetic model for the (−)cocaine–open channel interactions. The single channel data generated by simulation program (CSIM) according to this kinetic model adequately reconstructs the pH results (with a pK_a value of 7.1 for (−)cocaine, data not shown). We suggest that a more elaborate scheme (e.g., Schwarz et al., 1977), such as in situ deprotonation of charged cocaine or binding with the neutral cocaine form, is not required for the open Na^+ channel and cocaine interactions.

Why is the pK_a of cocaine near its binding site significantly different from the aqueous solution? The simplest explanation is that the environment near the binding site is very different from the aqueous solution (Fersht, 1984). Because the cocaine pK_a near its binding site becomes lower than that in aqueous solution, the charged cocaine has a tendency to lose its proton, suggesting that the conduction pathway near the cocaine binding site is relatively hydrophobic. This suggestion is not without precedent. It has been previously demonstrated that the LA binding site in acetylcholine receptor is also within the conduction pathway and the environment of this binding site appears to be relatively hydrophobic (for details, see Leonard et al., 1988; Miller, 1989).

Mechanism of the external pH effects on cocaine binding

One unexpected result in this report is the finding that raising pH_e enhances the apparent binding affinity of internal cocaine. This effect is not due to a direct action of

H^+ ions on the receptor site per se because pH_e has little effect on internally-applied quaternary local anesthetics.

What is the underlying mechanism for the pH_e effect on the binding of internal cocaine? Kinetic analyses show that the mean open time τ_o in the presence of cocaine is altered significantly by changes in pH_e, whereas mean closed time τ_c is little affected. An increase in pH_e decreases τ_o rather drastically, as if the concentration of the charged form of cocaine is raised near its binding site. How can pH_e influence the cocaine concentration in the channel pore to such a magnitude?

One possibility is that pH_e may modulate the local concentration of charged cocaine in the channel indirectly as described in Eqs. 8 and 9 and illustrated in Fig. 11. We know that according to Henderson-Hasselbach equation the higher the external pH the higher the concentration of the neutral form of cocaine at the external surface. The neutral form in the external unstirred layer can then enter the membrane phase which in turn can reequilibrate with neutral cocaine in the pore and in the internal unstirred layer. This reequilibration is dependent upon the concentration of neutral cocaine in aqueous phase as well as the partition and diffusion coefficients of neutral cocaine in the membrane. The end result is that the concentration of the neutral form of tertiary LA in the channel pore is higher when the pH_e is raised (Eqs. 6–9, also see Schwarz et al., 1977). This idea is further reinforced by the pH effects on externally applied cocaine. An increase in the pH_e drastically enhances the potency of external cocaine (Fig. 10), presumably due to a rapid increase in the membrane concentration of the neutral form which then escapes to the internal surface as well as the channel pore (Fig. 11 *A*). Apparently, the concentrations of cocaine in the membrane phase (neutral form), in the vicinity of external and internal surface of bilayers (both neutral and charged form within the unstirred layer), and in the channel pore reach a steady-state rapidly after solution stirring (Fig. 9). The relative potency difference for external cocaine between pH_e 6.2 and 8.4 is ~25-fold. This magnitude seems in agreement with the reported value for the relative concentration of radioactive procaine ($pK_a = 8.9$) that crosses the membrane from the external solution under various pH_e. Dettbarn et al. (1972) demonstrated that when squid giant axons were externally exposed to radioactive procaine for 10 min, the relative axoplasmic drug concentration was 17% for pH_e 5.8 and 627% for pH_e 9.0, a 37-fold difference.

It should be emphasized here that in intact tissue, external H^+ ions enter the channel pathway readily and appear to directly trap the LA drug longer in Na^+ channels as judged by the recovery time of the lidocaine-induced block (Schwarz et al., 1977; Chernoff, 1988). The pH in the channel pathway is believed to be equal to the pH_e when the gate is closed. In our bilayer study, the charged form can come and go through the internal hydrophilic pathway of BTX-activated Na^+ channels whereas in the normal Na^+ channels the access and the escape of the charged LA are presumably limited to the transient open state. We believe that such a physical difference is the basis for the different pH_e dependences in these two systems.

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REFERENCES

Aldrich, R. W., D. P. Corey, and C. F. Stevens. 1983. A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature (Lond.)*. 306:436–441.

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

Chernoff, D. M. 1988. Kinetics of local anesthetic binding to sodium channels: role of pK_a. Ph.D. thesis. Massachusetts Institute of Technology.

Dettbarn, W. D., E. Heilbronn, F. C. G. Hoskinn, and R. Kitz. 1972. The effect of pH on penetration and action of procaine ^{14}C, atropine ^{3}H, *n*-butanol ^{14}C and halothane ^{14}C in single giant axons of the squid. *Neuropharmacology*. 11:727–732.

Eigen, M., and G. G. Hammes. 1963. Elementary steps in enzyme reactions (as studied by relaxation spectrometry). *Adv. Enzymol.* 25:1–38.

Fersht, A. 1984. The pH dependence of enzyme catalysis. *In* Enzyme Structure and Mechanism. W. H. Freeman and Co., NY. 155–175.

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.

Green, W. N., L. B. Weiss, and O. S. Andersen. 1987. Batrachotoxin-modified sodium channels in planar lipid bilayers. Ion permeation and block. *J. Gen. Physiol.* 89:841–872.

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associates, Inc., Sunderland, MA. 272–302.

Katzung, B. G. 1982. Basic and Clinical Pharmacology. B. G. Katzung, editor. Lange Medical Publications, Ca. 2.

Khodorov, B. I. 1978. Chemicals as tools to study nerve fiber sodium channels: effect of batrachotoxin and some local anesthetics. *In* Membrane Transport Processes. Vol. 2. D. Tosteson, Y. Ovchinnikov, and R. Latorre, editors. Raven Press, New York. 153–174.

Krueger, B. K., J. F. Worley, and R. J. French. 1983. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. *Nature (Lond.)*. 303:172–175.

Leonard, R. J., C. G. Labarca, P. Charnet, N. Davidson, and H. A. Lester. 1988. Evidence that the M2 membrane spanning region lines the ion channel pore of the nicotinic receptor. *Science (Wash. DC).* 242:2578–2581.

Miller, C. 1989. Genetic manipulation of ion channels: a new approach to structure and mechanism. *Neuron.* 2:1195–1205.

Moczydlowski, E., and R. Latorre. 1983. Saxitoxin and ouabain binding activity of isolated skeletal muscle membrane as indicators of surface origin and purity. *Biochim. Biophys. Acta.* 732:412–420.

Moczydlowski, E., S. S. Garber, and C. Miller. 1984. Batrachotoxin-activated Na^+ channels in planar lipid bilayers. Competition of tetrodotoxin block by Na^+. *J. Gen. Physiol.* 84:665–686.

Moczydlowski, E., A. Uehara, X. Guo, and J. Heiny. 1986. Isochannels and blocking modes of voltage-dependent sodium channels. *Ann. NY Acad. Sci.* 479:269–292.

Moorman, J. R., R. Yee, T. Bjornsson, C. F. Starmer, A. O. Grant, and H. C. Strauss. 1986. pK_a does not predict pH potentiation of sodium channel blockade by lidocaine and W6211 in guinea pig ventricular myocardium. *J. Pharmacol. Exp. Ther.* 238:159–166.

Narahashi, T. 1971. Neurophysiological basis for drug action. Ionic mechanism, site of action and active form in nerve fibers. *In* Biophysics and Physiology of Excitable Membranes. W. J. Adelman, Jr., editor. van Nostrand-Reinhold, New York. 423–462.

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.

Nettleton, J., and G. K. Wang. 1989. Hydrogen ions affect the cocaine binding affinity in BTX-activated Na channels in planar bilayers. *Biophys. J.* 55:315a. (Abstr.)

Ritchie, J. M., and N. M. Greene. 1985. Local anesthetics. *In* The Pharmacological Basis of Therapeutics. A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murod, editors. MacMillan Publishing Co., New York. 302–321.

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

Starmer, C. F. 1986. Theoretical characterization of ion channel blockade: ligand binding to periodically accessible receptors. *J. Theor. Biol.* 119:235–249.

Wang, G. K. 1988. Cocaine-induced closures of single batrachotoxin-activated Na^+ channels in planar lipid bilayers. *J. Gen. Physiol.* 92:747–765.

Wang, G. K. 1989. Stereospecificity of a local anesthetic receptor in BTX-activated Na^+ channels. *Biophys. J.* 55:229a. (Abstr.)

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* 61:687–708.