Cocaine-induced Closures of Single Batrachotoxin-activated Na⁺ Channels in Planar Lipid Bilayers

GING KUO WANG
From the Department of Anesthesia Research Laboratories, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

ABSTRACT Batrachotoxin (BTX)-activated Na⁺ channels from rabbit skeletal muscle were incorporated into planar lipid bilayers. These channels appear to open most of the time at voltages > -60 mV. Local anesthetics, including QX-314, bupivacaine, and cocaine when applied internally, induce different durations of channel closures and can be characterized as "fast" (mean closed duration < 10 ms at +50 mV), "intermediate" (~80 ms), and "slow" (~400 ms) blockers, respectively. The action of these local anesthetics on the Na⁺ channel is voltage dependent; larger depolarizations give rise to stronger binding interactions. Both the dose-response curve and the kinetics of the cocaine-induced closures indicate that there is a single class of cocaine-binding site. QX-314, though a quaternary-amine local anesthetic, apparently competes with the same binding site. External cocaine or bupivacaine application is almost as effective as internal application, whereas external QX-314 is ineffective. Interestingly, external Na⁺ ions reduce the cocaine binding affinity drastically, whereas internal Na⁺ ions have little effect. Both the cocaine association and dissociation rate constants are altered when external Na⁺ ion concentrations are raised. We conclude that (a) one cocaine molecule closes one BTX-activated Na⁺ channel in an all-or-none manner, (b) the binding affinity of cocaine is voltage sensitive, (c) this cocaine binding site can be reached by a hydrophilic pathway through internal surface and by a hydrophobic pathway through bilayer membrane, and (d) that this binding site interacts indirectly with the Na⁺ ions. A direct interaction between the receptor and Na⁺ ions seems minimal.

INTRODUCTION
Local anesthetics reversibly inhibit Na⁺ currents in nerve and in muscle. Three complicated modes of local anesthetic action have been described so far, including tonic block of Na⁺ currents at the resting potential, use-dependent block of Na⁺ currents after repetitive depolarizations at constant voltages, and voltage-dependent block of Na⁺ currents after repetitive depolarizations at various voltages (for review see Hille, 1984; Strichartz and Ritchie, 1987). Despite numerous studies over the last

Address reprint requests to Dr. Ging Kuo Wang, Department of Anesthesia Research Laboratories, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115.
decade, the mechanism of local anesthetic action, however, remains unresolved. The main reason for this difficulty is that Na⁺ channels appear to have many transient conformations, which are known to be both voltage and time dependent. Conceptually, all these transient conformations may interact with the local anesthetic drug. Because of this complication several different hypotheses were proposed to explain the local anesthetic action on the inhibition of macroscopic Na⁺ currents (e.g., Strichartz, 1973; Khodorov et al., 1976; Hille, 1977; Hondeghem and Katzung, 1977; Starmer, 1987; Strichartz and Ritchie, 1987).

In contrast to many voltage- and time-dependent conformations of normal Na⁺ channels, it has been well documented that in planar bilayer batrachotoxin (BTX)-activated Na⁺ channels remain open most of the time at voltages larger than −60 mV (e.g., Krueger et al., 1983; Moczydlowski et al., 1984a). Furthermore, local anesthetics do not appear to bind to the BTX-binding site (Postma and Catterall, 1984). Accordingly, the blocking effects of local anesthetics may be observed in the bilayer system (Moczydlowski et al., 1986). Because of these two reasons, the planar bilayer system might provide a unique opportunity to investigate the detailed kinetics of open channel–local anesthetic interactions. In this report, we have focused on the mechanism of cocaine-induced closures of single BTX-activated Na⁺ channels in planar bilayers. Cocaine is the first local anesthetic used clinically (for history, see Ritchie and Greene, 1985). It contains a tertiary amine group with a pKₐ of 8.5 (Katzung, 1982). In addition to cocaine, when appropriate, we also show the results of two other local anesthetics, QX-314 and bupivacaine (pKₐ, 8.1), for comparison. Our results support the hypothesis that there is one single cocaine binding site on the BTX-modified Na⁺ channel and that quaternary amine local anesthetic QX-314 appears to compete directly for this binding site. The binding of cocaine is strongly voltage-dependent in bilayers for the open Na⁺ channels. In addition, external Na⁺ ions are found to reduce the cocaine-binding affinity significantly. To our surprise, internal Na⁺ ions display little effect on the cocaine binding.

**MATERIALS AND METHODS**

**Chemicals and Membrane Preparation**

Synthetic phospholipids, phosphatidylycholine (PC) and phosphatidylethanolamine (PE), were purchased from Avanti Polar Lipids (Birmingham, AL). BTX was a generous gift from Dr. John Daly, National Institutes of Health, Bethesda, MD. Bupivacaine-HCl and QX-314-chloride were gifts from Dr. Bertil Takman of Astra Pharmaceutical Products, Inc., Worcester, MA. Cocaine hydrochloride was purchased from Mallinckrodt, Inc., St. Louis, MO. The chemical structures of these local anesthetics are shown as follows:

![Chemical Structure of QX-314](image-url)
The bupivacaine and cocaine molecules contain chiral carbons (not shown). The bupivacaine drug used in this study is a mixture of stereoisomers, whereas the cocaine drug is the naturally occurring chemical, (-) form. Tetrodotoxin was obtained from Calbiochem-Behring Corp., La Jolla, CA. All other chemicals were reagent grade from commercial sources without further purification.

Plasma membrane vesicles were prepared from rabbit skeletal muscle as described by Moczydlowski and Latorre (1983), and Moczydlowski et al. (1984a). Light vesicles banding on a cushion of 30% sucrose (wt/vol) were diluted, pelleted, resuspended at ~10 mg protein/ml in 300 mM sucrose, and stored at -70°C.

Planar Bilayers and Na⁺ Channel Insertion

Planar bilayers were cast on 100–200-μm holes in polyvinyl chloride partitions from decane solutions containing 13.4 mg/ml PE and 6.7 mg/ml PC. Ionic currents were monitored at constant holding voltages, using low-noise electronics, EPC-7 list clamp (Medical Systems Corp., Great Neck, NY). Standard aqueous solution was 200 mM NaCl, 0.2 mM EGTA, 10 mM HEPES-NaOH, pH 7.4. Unless otherwise noted, both sides of the bilayer contained identical aqueous solutions. All other NaCl solutions also contained 0.2 mM EGTA and 10 mM HEPES-NaOH, pH 7.4.

Insertion of Na⁺ channels could be detected in the presence of 100 nM BTX added from the cis-side of the bilayer, essentially as described by Krueger et al. (1983). In general, plasma membrane vesicles (~10 μg/ml final concentration) were added to the cis side of the bilayer and the voltages were alternated between -50 and +50 mV every 20 s to facilitate their incorporation. All voltages were defined as intracellular voltage and the external face of Na⁺ channels in the bilayer was defined as zero voltage. These Na⁺ channels were blocked by tetrodotoxin (TTX) in a voltage-dependent manner, and were activated around -120 to -80 mV. The properties of rabbit BTX-activated Na⁺ channels appeared to be identical to those of rat counterparts (Moczydlowski et al., 1984a). Cocaine-HCl was dissolved in the standard aqueous solution at 100 mM stock concentration, aliquoted, and stored at -70°C until use. Stock solutions of QX-314 and bupivacaine were stored in -20°C. In general, local anesthetics were applied internally unless otherwise indicated. All experiments were performed at a room temperature of 25 ± 2°C. Currents were filtered at 50–100 Hz, recorded at 100-Hz resolution, stored and later analyzed by an AT computer using a pCLAMP software (Axon...
Instruments, Inc., Burlingame, CA) which uses Marquardt-Levenberg algorithms for statistical estimates of open and closed time constants. Bilayer records containing more than one channel were discarded.

RESULTS

**QX-314, Bupivacaine, and Cocaine Elicit Short, Intermediate, and Long Closures of Single Na⁺ Channels**

Single BTX-activated Na⁺ channels in planar bilayers are known to open most of the time at voltages > -60 mV (Krueger et al., 1983). Fig. 1 A shows the control records at ±50 mV. At this voltage range, brief spontaneous closures can be detected that account for only ~3% fractional time with no apparent voltage dependence (also see Moczydlowski et al., 1984a), whereas about 97% of the time the channels remain open.

When QX-314 is added to the internal side of Na⁺ channels, an apparent reduction in the unitary current flowing through open channels is observed (Fig. 1 B). This effect exhibits an apparent voltage dependence; the reduction of unitary conductance by QX-314 increases with increasing depolarizations. As a result, the ohmic I-V relation in the absence of drugs is altered to a rectifying I-V curve in the presence of QX-314 (Fig. 2). In addition, the current record shows a greater noise at +50 mV after QX-314 addition. These results are in agreement with a previous

![Figure 1: Local anesthetic-induced closures of single BTX-activated Na⁺ channels. (A) Examples of single-channel events in the absence of local anesthetic at ±50 mV. Records with brief closures were selected to indicate the current amplitude. The normally downward currents at -50 mV were displayed upward for comparison throughout this report. (B) After the addition of 5 mM QX-314. (C) After the addition of 300 μM bupivacaine. (D) After the addition of 300 μM cocaine. C and D are from different bilayers. Note that all three local anesthetics elicit stronger effects at the larger potential. Bilayers were formed in standard aqueous solution.](image-url)
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report by Moczydlowski et al. (1986). They suggested that this drug is a "fast" blocker, which exhibits on and off kinetics within 10–20 ms, so that the open channel is interrupted by the drug with an on-off flickering too fast to be resolved by our recording resolution.

In contrast, both bupivacaine and cocaine induce clear closing events (Fig. 1, C and D). The mean duration of these closing events at +50 mV is measured to be 403 and 80 ms for cocaine and bupivacaine, respectively; the mean open duration is 110 ms and 317 ms, respectively. Thus, based on the dwell time of closures, bupivacaine is considered to be an "intermediate" blocker and cocaine a "slower" one. The amplitude of the Na⁺ channel unitary conductance, however, is slightly altered by bupivacaine or cocaine at moderate concentrations (Fig. 3, left panel). A small reduction of the mean unitary current (mean amplitude: bupivacaine, 0.91 pA; cocaine, 0.85 pA; control, 1.05 pA at +50 mV) is observed. As the open duration decreases, the averaged current amplitude is more likely to be reduced (Fig. 3, right panel). Since brief closures of <30 ms were included in the opening event (see below), the averaged current amplitude of openings is biased and may appear to be

Figure 2. (A) Current-voltage relations before and after the addition of internal or external 5 mM QX-314. (O) Before QX-314, (a) after external QX-314, and (■) after internal QX-314. Current amplitudes of open events within a 1-min record were measured and averaged. Brief closures of <30 ms were not registered as closures. This bilayer was formed in standard aqueous solution. (B) Voltage dependence of the intracellular QX-314 block. The data (■) from A at each voltage were averaged and used to obtain the KD value according to the equation 

\[
KD = \frac{[QX-314] \cdot i_o}{i_o - i_b},
\]

where [QX-314] is 5 mM, \(i_o\) is the current amplitude in the absence of blocker, and \(i_b\) is the averaged current amplitude in the presence of internal QX-314. The solid line is drawn by eye according to the equation

\[
KD = KD(0 \text{ mV}) \cdot \exp \left( -\delta \Delta V / kT \right),
\]

where \(KD(0 \text{ mV})\) is estimated to be 6.6 mM, \(\delta\) is an estimated equivalent valence of 0.46 (0.48 ± 0.02, \(n = 3\)), \(\Delta V\) is the membrane potential difference, \(e\) is the elementary charge, \(k\) is Boltzmann's constant, and \(T\) is the temperature in Kelvin.
less than the true value of the unitary conductance. At high cocaine concentrations (>1 mM), the single-channel conductance cannot be measured meaningfully because of the extremely short opening events. These results suggest that local anesthetics do not affect the single-channel amplitude. Instead, the observed reduction can be attributed to the limited bilayer resolution.

The action of bupivacaine and cocaine appears to be strongly voltage-dependent; higher voltages give rise to stronger blocking effects (Fig. 1). This voltage depen-

![Amplitude histograms and open duration vs. amplitude plots for bupivacaine and cocaine](image)

**Figure 3.** (A and B, left panel) Amplitude histograms of BTX-activated Na$^+$ channels at +50 mV in the presence of internal bupivacaine or cocaine (300 μM). The amplitude of each opening event was determined by averaging all data points in the opening event. An open event was registered when the amplitude of the current reached a threshold set at half of the averaged current amplitude, ~0.5 pA. After the opening, the duration of an open event was measured when the channel closed below threshold for at least 30 ms. Brief closures of <30 ms were not counted as closures. Opening events that lasted only 10 ms were arbitrarily set as zero current. Bilayers were formed in standard aqueous solution. (A and B, right panel) Open duration vs. amplitude plots. The amplitude determined in each open event are plotted against the open duration. As the open duration becomes shorter, the averaged amplitude of such an event also becomes smaller. Only 150 events are shown for bupivacaine and cocaine, both applied internally at 300 μM. The membrane potential was +50 mV.

dence of the closing events seems comparable with the voltage dependence of the current reduction by QX-314, and is opposite to that of benzocaine and procaine reported previously (Moczdolowski et al., 1986). In the remaining section, the action of cocaine will be described in detail since cocaine appears to be the most potent drug among the various other local anesthetics tested so far in bilayers, including lidocaine, etidocaine, GEA-968, procaine, and benzocaine.
Equilibrium Cocaine Binding Indicates a Single Binding Site Present in the Na⁺ Channel

To characterize the drug-binding site(s), the reduction of the open probability by cocaine is analyzed at various cocaine concentrations. Fig. 4, A and B show the blocking events and the dose-response curves of cocaine on the open probability of Na⁺ channels, from 10 μM up to 3 mM. At this concentration range, the fractional closed time can be well described by the Langmuir isotherm:

\[ f_c = \frac{[L]}{K_{D(app)} + [L]} \]  

where \( f_c \) is the fractional closed time \((1 - f_o, \text{ where } f_o \text{ is the fractional open time})\), \([L]\) is the concentration of cocaine, and \(K_{D(app)}\) is the apparent dissociation constant.

![Figure 4](image-url)

**Figure 4.** Dose-response curves for cocaine-induced closures. (A) Examples of single-channel current traces at +50 mV with various internal cocaine concentrations in standard aqueous solution. Total open times decrease as the cocaine concentration is raised from 10 μM up to 3 mM. (B) The fractional open time \((f_o)\) is plotted against the cocaine concentration. The solid line is drawn according to Eq. 1 and is fitted by eye. \(K_{D(app)}\) for cocaine at +50 mV is 80 μM, \(K_{D(app)}\) at -50 mV is 600 μM. Bilayer membranes were formed in standard buffer. Each data point represents the fractional open time of a 5- or 10-min continuous record containing >500 opening events.

of cocaine concentration at which \(f_c = 0.5\). The \(K_{D(app)}\) at -50 mV is calculated to be 600 μM, about eightfold higher than the \(K_{D(app)}\) at +50 mV, which is measured at ~80 μM. These data suggest that the blocking events after the cocaine addition are due to the binding of one cocaine molecule to one Na⁺ channel, and that the binding affinity is strongly voltage dependent. Further kinetic analyses will follow to substantiate these notions.

**Kinetics of Cocaine-induced Channel Closures**

In Fig. 5, A and B, open-time and closed-time histograms after the internal cocaine addition are presented. Despite the exclusion of ~85% of fast spontaneous closing
events with the cut-off time of 30 ms, the histograms presumably still contain the intrinsic spontaneous closing events, estimated to be ~2.5% of the cocaine-induced total events in a 5-min recording time. The spontaneous closing events in this voltage have closed-time constants of 7.8 and 102 ms (see Moczydlowski et al., 1984a). Consequently, the kinetic data in this report are contaminated with these intrinsic closing events. This error can not be easily corrected because the intrinsic closing events overlap with the cocaine-induced closing events. However, since the intrinsic closing events are rare, the closed-time and open-time histograms in the presence of cocaine can still be well fitted with a single exponential at the various voltages.

![Figure 5](image)

Figure 5. Kinetic properties of cocaine-induced closures on single BTX-activated Na⁺ channels. (A) Distribution of open time. Open-time intervals were measured in standard aqueous solution with 300-µM internal cocaine at +50 mV in a 5-min record containing 598 events. All observations longer than 30 ms were fitted by the method of nonlinear least-square (pSTAT Software of Axon Instruments, Inc.) with a distribution described by a one-exponential term: 

\[ N(t) = N \cdot \exp\left(-t/\tau_o\right) \]

where \( N(t) \) is the number of open events of duration \( t \), \( N \) is the number of events in the population at \( t = 0 \), and \( \tau_o \) is the mean open time; \( N = 187, \tau_o = 106 \text{ ms} \). (B) Distribution of closed time. The same membrane as A. All observations longer than 60 ms were fitted with the equation, 

\[ N(t) = N \cdot \exp\left(-t/\tau_c\right) \]  

\( N = 116, \tau_c = 337 \text{ ms} \). In several experiments under the same conditions, the \( \tau_o \) and \( \tau_c \) values were 96.8 ± 14.3 and 367.0 ± 48.7 ms (n = 5), respectively.

tested, which suggests that most events (>95%) are caused by a binding reaction between cocaine and the Na⁺ channel. The cocaine-binding reaction may be described as the following:

\[ O + L \xrightarrow{k_1} O \cdot L, \quad (\text{Scheme A}) \]

where \( O \) stands for open channel, \( L \) for local anesthetic cocaine, \( O \cdot L \) for the cocaine-bound nonconducting Na⁺ channel, \( k_1 \) for the rate constant for cocaine association, and \( k_{-1} \) for the rate constant for cocaine dissociation from the channel.
The rate constants can be related to the open ($r_o$) and closed ($r_c$) time distribution:

$$k_1 = 1/(r_o[L])$$  \hspace{1cm} (2)

$$k_{-1} = 1/r_c$$  \hspace{1cm} (3)

and the cocaine dissociation constant is calculated as:

$$K_D = k_{-1}/k_1.$$  \hspace{1cm} (4)

For scheme A to be correct, it is necessary to establish that $1/r_o$ is concentration dependent, whereas $1/r_c$ is not. Fig. 6, A and B show that for the best approximation this is the case. At $\pm 50$ mV, the $1/r_o$ value increases as a linear function of the cocaine concentration, whereas $1/r_c$ is essentially constant. This result thus agrees well with the prediction of scheme A. The $K_D$ values determined by the kinetic analyses (for example, $K_D = 94 \mu M$ at $+50$ mV; in six separate experiments, $K_D = 75.5 \pm 17.3 \mu M$) are also consistent with equilibrium binding results ($80 \mu M$ at $+50$ mV). We conclude that cocaine is an open-channel blocker that apparently binds with the Na$^+$ channel in a one-to-one relationship.

**Voltage and Na$^+$ Ion Dependence of Cocaine Binding**

Fig. 7 shows the cocaine-binding rate constants measured over a range of voltages at 100 and 500 mM Na$^+$ concentrations. Both rate constants vary exponentially with voltage, with $k_1$ increasing e-fold per 73 mV (Fig. 7 A), and $k_{-1}$ decreasing e-fold per 119 mV (Fig. 7 B). Furthermore, these rate constants are [Na$^+$] dependent. The association rate constants are greatly reduced by about fourfold when the Na$^+$ ions...
are raised from 100 to 500 mM, which suggests that Na⁺ ions may compete with the cocaine-binding site. However, one unexpected result that deviates from a strict competition scheme between Na⁺ and cocaine for the binding site is that \( k_{-1} \) is also increased by raising Na⁺ ions. Evidently, Na⁺ ions enhance the dissociation of cocaine from its binding site, which demonstrates that secondary interactions between Na⁺ ions and the cocaine-binding site have occurred. Fig. 8 shows the Na⁺ dependence of \( K_D \) measurements at various voltages. Higher Na⁺ ions reduce the binding affinity, whereas higher voltages increase the binding affinity. An increase of voltage by 44 mV decreases the \( K_D \) value by e-fold. This curve can be fitted by the equation:

\[
K_D(\Delta V) = K_D(0 \text{ mV}) \cdot \exp(-\delta \Delta V e/kT)
\]

where \( K_D(0 \text{ mV}) \) is the estimated \( K_D \) at 0 mV, \( \delta \) is an equivalent valence that quantifies how the applied voltage (\( \Delta V \)) affects cocaine binding, \( e \) is the elementary charge, \( k \) is Boltzmann's constant, and \( T \) is the temperature in Kelvin. The \( \delta \) value is estimated to be 0.59 for cocaine at both 100 and 500 mM [Na⁺]. In several separate experiments, the mean \( \delta \) value is 0.55 ± 0.07 (SD, \( n = 5 \)). This value is slightly larger
than the δ value for QX-314 (δ = 0.48 \pm 0.02, n = 3, Fig. 2) and close to the value for bupivacaine (δ = 0.58, n = 2).

**The Effects of External and Internal Na⁺ Ions on the Cocaine Binding**

Since raising the symmetrical Na⁺ ion concentrations reduces the cocaine-binding affinity significantly, it seems appropriate to study the effects of Na⁺ ions at asymmetrical conditions so that the mechanism of this phenomenon can be better understood. To our surprise, the internal Na⁺ ions appear to have little effect on the cocaine binding. Increase of the internal Na⁺ ions from 40 to 200 mM (while keeping the external [Na⁺] constant at 200 mM) does not reduce the binding affinity of cocaine. The Kᵦ value, the k₋₁, and the k₁ values remain nearly identical to those measured at 200 mM symmetrical Na⁺ ions (Fig. 9). Increasing the internal Na⁺ ions further, from 100 up to 400 mM in two separate experiments (while keeping the external [Na⁺] constant at 500 mM), also fails to significantly change the kinetic values at V = 0 mV among these conditions.

In contrast, an increase of the external Na⁺ ions from 100 to 300 mM drastically alters the cocaine-binding kinetics (Fig. 10). The cocaine dissociation rate constant (k₋₁) is raised by the increase of Na⁺ ions, whereas the association rate (k₁) is reduced. In general, the association rate is affected more than the dissociation rate by external [Na⁺]. It is usually easier to detect the differences in the k₁ than in the k₋₁ (Figs. 7 and 10; k₋₁ = 2.6 \pm 0.6 s⁻¹ [n = 4], 3.7 \pm 1.0 s⁻¹ [n = 4], and 4.9 \pm 0.6 s⁻¹ [n = 5] estimated at V = 0 mV for external 100, 300, and 500 mM [Na⁺], respectively). The calculated Kᵦ value is increased by almost sixfold. The kinetics of
cocaine binding in the 300 mM Na⁺ (out)/100 mM Na⁺ (in) condition are therefore almost identical to that in 300 mM symmetrical Na⁺ ions. These results demonstrate that the binding of cocaine can be strongly influenced by the external Na⁺ ions but not by the internal Na⁺ ions.

**QX-314 and Cocaine Binding Competition**

Because of the similar voltage dependence of QX-314 and cocaine in the reduction of the Na⁺ current amplitude and in the induced-closure events, respectively, it is reasonable to suspect that these two local anesthetics may compete with the same binding site. When 5 mM QX-314 was added to the internal solution containing 300 μM cocaine, the single Na⁺ channel conductance at +50 mV was reduced and the current was noisier as to the cocaine-treated counterpart (Fig. 11, A and B). In addition, the open duration of the single channel was significantly lengthened by QX-314 (Fig. 11, C and D). The closed durations, however, remained unchanged and their time constants were measured at 2.34 s⁻¹ for cocaine alone and 2.24 s⁻¹ for cocaine plus QX-314 in the same bilayer at +50 mV.

Also as expected, QX-314 at -50 mV did not compete as strongly with cocaine as at +50 mV. The τc value with the cocaine alone was only increased by a factor of 1.36 at -50 mV by 5 mM QX-314, whereas at +50 mV it was increased by a factor of 2.95. In contrast, the τc value was little changed by QX-314 whether the voltage was at +50 or -50 mV. These results demonstrate that QX-314 and cocaine compete with the same binding site and is consistent with the following simple kinetic
where O is the conducting Na⁺ channel. Both cocaine and QX-314 compete with the same binding site and it is mutually exclusive. When the channel is occupied by a cocaine molecule, QX-314 cannot reach its binding site; thus, the dissociation rate constant for cocaine is not changed. Similarly, when the channel is occupied by a QX-314 molecule, cocaine has to wait for QX-314 unbinding. As a result, the association rate constant for cocaine is decreased by QX-314 (Fig. 11). Since the on and off kinetics for QX-314 are extremely fast while the channel is not bound with cocaine, no normal Na⁺ currents are detected. From scheme B, an equation can
also be further derived to calculate the $K_D$ values of QX-314:

$$
\tau_o(V)_{\text{app}} = \tau_o(V) \left( 1 + \frac{[\text{QX-314}]}{K_D(V)} \right),
$$

where $\tau_o(V)_{\text{app}}$ and $\tau_o(V)$ are the apparent open time constants at voltage $V$ in the presence of cocaine plus QX-314, and cocaine alone, respectively; $[\text{QX-314}]$ is the concentration of QX-314 applied; $K_D(V)$ is the dissociation constant for QX-314 at voltage $V$. The calculated $K_D$ values for QX-314 are 13.9 mM at -50 mV and 2.6 mM at +50 mV. These values are in good agreement with the directly measured $K_D$ values shown in Fig. 2 B.

**FIGURE 11.** Competition between QX-314 and cocaine. Examples of single-channel records at +50 mV in the presence of (A) 300 μM cocaine and (B) in the presence of both 300 μM cocaine and 5 mM QX-314. Both drugs were applied from inside. (C) Distribution of open time with 300 μM cocaine. The data were measured as described in Fig. 5. $N = 248$, $\tau_o = 78$ ms. (D) Distribution of open time with 300 μM cocaine and 5 mM QX-314. $N = 105$, $\tau_o = 151$ ms. Bilayer membrane was formed in a 200-mM symmetrical NaCl solution.

**External Vs. Internal Applications of Local Anesthetic QX-314, Bupivacaine, and Cocaine**

QX-314 is a quaternary local anesthetic with a permanent charge. This charged molecule does not pass through the membrane nor does it pass through the channel ion pathway judging by the fact that external application of QX-314 has no effects on the single-channel conductance (Fig. 2). On the other hand, bupivacaine and cocaine are both lipid-soluble drugs and can easily pass through the membrane barrier (Ritchie and Greene, 1985). Consequently, external applications of bupivacaine
and cocaine are almost as effective as internal applications. The potency of drugs, i.e., the $K_D$ value estimated by $k_{-1}/k_1$, is comparable between internal and external applications. For example, in two separate experiments the average $K_D$ value for externally applied cocaine is $118 \mu M$ at $+50$ mV in the 200-mM symmetrical NaCl concentration, which is quite close to the value of internally applied cocaine ($76 \mu M$). These results suggest that both hydrophobic and hydrophilic pathways exist for tertiary amine local anesthetics. Only the hydrophilic pathway is present for the quaternary amine derivative QX-314.

The externally applied cocaine molecule appears to bind to the same binding site as the internally applied cocaine molecule. As a piece of evidence, external Na$^+$ ions are found to compete effectively with cocaine binding even when the drug is applied from the outside. Similarly, internal QX-314 also competes strongly with the externally applied cocaine molecule. These results further confirm that there is only one single class of binding site for the cocaine molecule, and this binding site can be reached by a hydrophobic or hydrophilic pathway.

**DISCUSSION**

**One Cocaine Molecule Closes One Na$^+$ Channel**

This report demonstrates that local anesthetics, such as cocaine and bupivacaine at moderate concentrations, close the channels in an all-or-none manner. Although QX-314 reduces the single-channel conductance and elicits a higher current noise, these effects are present only at high concentrations and are probably due to the fast dissociation rate of this drug, which exceeds our recording resolution. The result obtained from the cocaine dose-response curve strongly indicates that one cocaine molecule closes one Na$^+$ channel. A common local anesthetic binding site is sufficient to explain our kinetic data from bilayers with externally and internally applied cocaine, which display a single-exponential distribution for both closed and open times. This phenomenon is in agreement with the hypothesis that both charged and neutral forms of tertiary-amine local anesthetics can reach this common receptor site through hydrophilic and hydrophobic pathways (Hille, 1977).

**Voltage-dependent Binding of Local Anesthetics**

Ever since the report of Strichartz (1973), who first demonstrated the voltage-dependent inhibition of Na$^+$ currents by QX-314 in frog myelinated nerve, many local anesthetics (but not all) have been characterized to exhibit a similar strong voltage-dependent behavior. As a rule, larger repetitive depolarizations cause greater inhibition of Na$^+$ currents by this type of local anesthetics. Such a phenomenon appears to also be present in the BTX-treated Na$^+$ channels, despite that local anesthetics bind to the BTX-treated channel with lesser affinities as measured by electrophysiological and biochemical methods (Khodorov, 1978; Postma and Catterall, 1984). Somewhat surprisingly, Moczydlowski et al. (1986) found that benzocaine as well as procaine induce long-lived blocking events more effectively at low voltages than at high voltages (measured between $-70$ to $+50$ mV). In addition, they observed that these two local anesthetics appear to bind preferentially to a closed state of the BTX-treated channel. (Procaine also reduces the Na$^+$ conductance but
in a voltage-dependent manner similar to that of QX-314.) This opposite voltage-
dependence of local anesthetic inhibition of Na\(^+\) currents in bilayers has not been
described in normal Na\(^+\) channels (Hille, 1984). Whether or not these two different
blocking modes are mediated via different anesthetic binding sites remains to be
established.

**Mechanisms of Voltage-dependent Binding**

The voltage dependence of the cocaine binding exhibits an effective valence of \(\delta = 0.55\) (Fig. 8). This value is comparable to that for bupivacaine (\(\delta = 0.58\)) and for
QX-314 (\(\delta = 0.48\) from this report; also see Strichartz, 1973; Moczydlowski et al.,
1986). In a model proposed by Strichartz (1973), this parameter was taken to reflect
the depth of drug penetration influenced by the applied field. If true, our results
indicate that all three local anesthetics can enter the Na\(^+\) channel as deep as half of
the electric field. Consistent with this model is that the rate constants for cocaine
association (\(k_i\)) and dissociation (\(k_{-i}\)) are both voltage dependent. The association of
drug with the binding site is favored by depolarization, whereas the dissociation is
favored by hyperpolarization.

Although our results agree with the hypothesis that local anesthetics move
through the electric field to reach their binding site, they by no means exclude other
possible mechanisms for the local anesthetic action. We now discuss these alterna-
tives. It has been reported that in normal and chloramine-T-treated squid axons the
apparent voltage-dependent inhibition of Na\(^+\) currents by QX-314 reaches an
asymptote at voltages above \(+50\) mV (Cahalan and Almers, 1979; Wang et al.,
1987). However, in two preliminary experiments in bilayers, we found no evidence
of such a phenomenon at voltages of up to \(+100\) mV; the \(K_d\) values for cocaine
continue to decrease as the voltage increases. The reason for this difference in nor-
mal and BTX-treated Na\(^+\) channels is not clear. Further studies are needed to
extend the measurements of voltage-dependent binding to voltages beyond \(+100\)
mV. This test is particularly critical in determining the origin of voltage-dependent
binding. If binding rate constants should saturate at high positive and negative volt-
ages, this would imply that the receptor conformation can be an important deter-
ninant for voltage-dependent binding (for discussion see Moczydlowski, 1984b;
Wang et al., 1987).

In addition to the saturation of the voltage-dependent block in intact tissue at
high voltages, two major differences between control and BTX-activated Na\(^+\)
channels need to be considered. First, Na\(^+\) channel activation occurs normally be-
 tween \(-40\) and \(+20\) mV (Hodgkin and Huxley, 1952) but occurs between \(-120\)
and \(-80\) mV for BTX-activated Na\(^+\) channels. Since the activation “gate” may limit
the access of the drug to its receptor (Strichartz, 1973), the voltage-dependent block
in intact tissue needs to include this factor (Yeh and Tanguy, 1985; Strichartz and
Wang, 1986; Starmer, 1987; Wang et al., 1987). In planar bilayers, the channel is
open most of the time between \(-50\) and \(+50\) mV. The apparent voltage-dependent
binding of cocaine and bupivacaine in bilayers, therefore, reflects exclusively the
open channel/local anesthetic interactions. Second, BTX may bring about a new
voltage-dependent binding of local anesthetics. If true, this would resemble the
newly uncovered TTX voltage-dependent binding in BTX-activated Na\(^+\) channels.
So far, no direct voltage-dependent binding was found between TTX and normal Na\(^+\) channels in nerves (Rando and Strichartz, 1986). Based on these considerations, the mechanism of the voltage-dependent binding in intact tissue and in bilayers may be considerably different.

**Is the Cocaine-binding Site Within the Pore?**

The strong voltage dependence of cocaine binding seems to support that its receptor is deeply within the ion conduction pathway (French and Shoukimas, 1985). However, as discussed above, this evidence alone may be insufficient to make a firm conclusion. For example, in lipid bilayers TTX also exhibits a similarly strong voltage-dependent binding, but its binding site is proposed to locate superficially on the Na\(^+\) channel's external surface and not deep within the channel pore (Moczydowski et al., 1984b; Green et al., 1987).

More directly, in this report we have shown that QX-314 competes with cocaine for its binding site in a mutually exclusive manner. Since QX-314 carries a permanent charge, it does not penetrate deeply to the hydrophobic area. Based on the internal QX-314 block of Na\(^+\) currents in nerve, it has been suggested that the QX-314-binding site is located in the pore between the selectivity filter and the physical gate(s) (Strichartz, 1973; Hille, 1984). Taken together, our results therefore suggest that the cocaine-binding site is also located within the aqueous pore that can be reached by QX-314 only when this drug is applied internally. In addition, the fact that external but not internal Na\(^+\) ions reduce the cocaine binding affinity gives further strong support for such an interpretation. This Na\(^+\) ion effect will be discussed in more detail in the following text.

**External Na\(^+\) Ions Reduce the Binding Affinity of Cocaine**

Our results demonstrate that the external Na\(^+\) ions have strong effects on the cocaine-binding interactions (Fig. 10). An increase of the external Na\(^+\) ion concentration from 100 to 300 mM reduces the binding affinity by 5.5-fold. These data are in agreement with the report of Cahalan and Almers (1979) who have shown that after the removal of Na\(^+\) ions from external solution the blocking effect of internally applied QX-314 is enhanced, which demonstrates the antagonism between external Na\(^+\) ions and internal QX-314. In bilayers, the QX-314 effect is also enhanced by about twofold when the external Na\(^+\) ion concentration is reduced from 200 to 100 mM (data not shown).

What is the mechanism underlying the antagonistic effect between external Na\(^+\) ions and the local anesthetics? The kinetics of the cocaine-binding interactions under various external Na\(^+\) ion concentrations may provide some clues. A strict competition between Na\(^+\) ions and the cocaine molecules for the cocaine-binding site will reduce the cocaine-binding affinity. However, under the strict competition scheme, both internal or external Na\(^+\) ions should not affect the dissociation rate constant of cocaine (i.e., \(k_{-1}\), scheme A), but only reduce the cocaine association rate constant (\(k_1\)). This is not what we have observed. Both the cocaine association and dissociation rate constants are changed by the external Na\(^+\) ions but not by the internal Na\(^+\) ions. As a comparison, the internal QX-314 was found to compete with cocaine at a mutually exclusive manner (scheme B). Only the cocaine association...
rate was reduced; the dissociation rate was not altered by the internal QX-314. These results thus suggest that either electrostatic repulsion (i.e., a "knock-out" phenomenon) or some indirect interactions occur between Na\(^+\) ions and the cocaine-binding site.

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