Binding Affinity and Stereoselectivity of Local Anesthetics in Single Batrachotoxin-activated Na⁺ Channels

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ABSTRACT Several local anesthetics (LA) have been previously shown to block muscle batrachotoxin (BTX)-activated Na⁺ channels in planar bilayers. The mean dwell time of different LA drugs, however, varies widely, from <10 ms to longer than several seconds. In this study, we have examined the structural determinants that govern the dwell time, the binding affinity, and the stereoselectivity of LA drugs using cocaine and bupivacaine homologues, RAC compounds, and their available stereoisomers. Our results from the structure–activity experiments reveal that (a) there are two apparent hydrophobic binding domains present in the LA binding site; one interacts with the aromatic moiety of the LA drugs, and the other interacts with the alkyl group attached to the tertiary amine of the LA drugs; (b) the LA mean dwell time and the binding affinity are largely determined by the hydrophobic interactions; (c) the LA binding site is highly stereoselective, with a difference in Kᵩ values over 50- and 6-fold for (+/-) cocaine and (+/-) bupivacaine, respectively; (d) the cocaine stereoselectivity is comparable among muscle, brain, and heart BTX-activated Na⁺ channels; and finally and most unexpectedly, (e) the stereoselectivity of LA drugs in BTX-activated Na⁺ channels appears greatly different from that reported in normal Na⁺ channels. Possible explanations for this difference are discussed.

INTRODUCTION Local anesthetics (LA) are a group of drugs that reversibly block the propagation of action potentials in excitable membranes (Ritchie and Greene, 1985). These drugs are known to interact directly with the voltage-gated Na⁺ channel and upon binding to inhibit Na⁺ ions from passing through the channel. The potency or the binding affinity of different LA, however, varies widely over three orders of magnitude, ranging from micromolar to millimolar concentration (Courtney, 1980). The molecular basis for this variation remains to be studied.

Recently, an LA binding site was identified in the batrachotoxin (BTX)-activated Na⁺ channels in planar bilayers for both tertiary and quaternary amine local
anesthetics (Moczydlowski et al., 1986b; Wang, 1988). This binding site appears to be within the pore and its interaction with local anesthetics is strongly voltage-dependent. Furthermore, different LA drugs induce very different mean durations of closures ranging from <10 ms to more than several seconds. The reason for this large variation of LA dwell time is not known. In order to characterize this LA binding site in more detail, we have applied a series of LA drugs and examined their binding interactions with BTX-activated Na+ channels in planar bilayers. Among the drugs surveyed are: (a) cocaine homologues which include (+),(-)cocaine, (-)norcocaine, (+),(-)pseudococaine, ecgonine methylester; (b) bupivacaine homo-

![Figure 1](image.png)

**FIGURE 1.** Basic chemical structures of several LA drugs used in this report. The substitution of R = -C4H9 attached to the tertiary amine yields bupivacaine homologues (left). Cocaine homologues (middle) are compounds with different substitutions on the tropane ring. All cocaine homologues in this study have the O-benzoyl moiety at the equatorial position. RAC421 is a quaternary derivative of RAC109 with an additional ethyl group on the tertiary amine (right). Because of a permanent charge, the RAC421 compound is inactive in bilayers when applied externally to the Na+ channel.

logues which include (+),(-)mepivacaine, (+),(-)ropivacaine, (+),(-)bupivacaine, RAD-395, and 1-octyl-2',6'-pipécoloxylidide; and (c) RAC compounds such as RAC421 (I) and (II) and RAC109 (I) and (II). The chemical structures of eight representative drugs are shown in Fig. 1. Detailed structures of cocaine homologues can be found in Matthews and Collins (1983). The relevant chiral carbons are asterized to indicate the location of the R (rectus) and S (sinister) forms according to Cahn-Ingold-Prelog configuration system. It is noteworthy that not all S forms of LA drugs correspond to (-) forms.
Stereoisomers have been known to be excellent probes for the topology of receptor-ligand complexes (Fersht, 1985). Such an approach to study LA receptor topology has also been taken by examining the effect of different RAC stereoisomers on macroscopic Na\(^+\) currents (Hille et al., 1975; Yeh, 1980). The bilayer system was chosen in this study because of its simplicity and easy drug access. Furthermore, the bilayer system can be applied to study single Na\(^+\) channels from various tissue sources with identical ionic conditions, lipid compositions, and pH. Our studies on the structure-activity relationship suggest that there are two hydrophobic domains at the local anesthetic binding site. We have further found that the potency of these drugs in bilayers is largely determined by the drug dwell time, which in turn is determined by the hydrophobic interactions. Furthermore, the LA binding site in BTX-activated Na\(^+\) channels appears to be relatively conserved in muscle, brain, and heart tissues as judged by their comparable cocaine stereoselectivity. Curiously and most unexpectedly, BTX appears to alter the LA stereoselectivity of normal Na\(^+\) channels reported in the literature. Preliminary results of this report have appeared elsewhere (Wang, 1989).

**MATERIALS AND METHODS**

**Chemicals**

Cocaine and its derivatives were obtained from Dr. Rao Rapaka, Research Technology Branch, National Institute of Drug Abuse, Bethesda, MD. All bupivacaine homologues and RAC stereoisomers were gifts from Dr. Bertil Takman of Astra Pharmaceutical Products, Inc., Worcester, MA and Dr. Rune Sandberg of Astra Pain Control, Sodertalje, Sweden. Synthetic phosphatidylcholine (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. Tetrodotoxin was obtained from Calbiochem-Behring Corp., La Jolla, CA. All other chemicals were reagent grade from commercial sources without further purification.

**Plasma Membrane Preparation**

Plasma membrane vesicles from rabbit skeletal muscle were prepared according to the method of Moczydlowski and Latorre (1983). Sarcolemmal membranes from calf ventricular muscle were prepared by using the method of Guo et al. (1987). Membrane vesicles from rabbit brain were prepared as previously described by Moczydlowski et al. (1986a). All membrane isolation methods were performed at 0–4°C. Each preparation was suspended in 0.3 M sucrose, 10 mM HEPES, 0.2 mM EDTA, and 0.02% NaN\(_3\), aliquotted, and stored at −70°C.

**Planar Bilayers and Na\(^+\) Channel Insertion**

Planar lipid bilayers were formed on ~200-μm holes in polyvinyl chloride partitions from decane solution containing 13.4 mg/ml PE and 6.7 mg/ml PC. Standard aqueous buffer was 200 mM NaCl, 0.2 mM EGTA, 10 mM HEPES-NaOH, pH 7.4 for both cis and trans sides. Plasma membrane vesicles (~10–50 μg/ml final concentration) were added to the cis side of the bilayer. Insertion of Na\(^+\) channels could be detected in the presence of 100 nM BTX, also added to the cis side of the bilayer, essentially as described by Krueger et al. (1983). To facilitate vesicle incorporation, alternative pulses of −50 and +50 mV with 10-s duration were applied to the bilayers. Cocaine-HCl and its derivatives were dissolved in standard aqueous
buffer at 100–200 mM stock concentration, aliquotted, and stored at −70°C until use. Stock solutions of bupivacaine, mepivacaine, ropivacaine, RAD-393, and RAC compounds were prepared in standard aqueous buffer and stored at −20°C. 1-Octyl-2',6'-pipicoloxylidide was dissolved in 50% ethanol and stored at −20°C. In general, local anesthetics were applied to the internal side of Na⁺ channels. The external side of Na⁺ channels could be determined by its sensitivity to tetrodotoxin (TTX) at the end of experiments, although high concentration (~1 μM) is required to detect the block on calf ventricular Na⁺ channels (Guo et al., 1987) which is about 50–100 times higher than the concentration required for rabbit skeletal muscle and brain Na⁺ channel subtypes. Alternatively, the side of Na⁺ channels can be determined by the channel gating around −100 mV (Krueger et al., 1983; Moczydlowski et al., 1984).

**Na⁺ Current Recording and Data Analysis**

Ionic currents were monitored at constant holding voltages using EPC-7 List clamps (Medical Systems Corp., Great Neck, NY). Currents were normally low-pass filtered at 100 Hz unless indicated otherwise (cutoff frequency, fc = −3 db; model 3200, Krohn-Hite Corp., Avon, MA), recorded at a sampling rate of 100 or 400 Hz, stored and later analyzed by an IBM-AT computer using a pCLAMP software with a Labmaster data acquisition board (Axon Instruments, Inc., Burlingame, CA). Bilayer baseline noise was typically ~0.15–0.25 pA under our recording conditions. When a drug induces channel closures with a mean closed time of longer than 50 ms, data were acquired at 100 Hz and analyzed as described before (Wang, 1988). When the drug-induced closed times had a mean duration between 10 and 50 ms, data were acquired at either 100 or 400 Hz for analysis. Data acquired and analyzed at these two sampling rates gave comparable results. Throughout this report, all data analyses were performed using pCLAMP software and the threshold for open channel detection at +50 mV was set ~0.5 pA above the baseline. When drug-induced closing events could not be resolved, open channel amplitude was determined by averaging the Na⁺ current within a 1–2 min record. Bilayers containing more than one Na⁺ channel were not used in this report.

**RESULTS**

**Structure-Activity Relationship of Cocaine Homologues in BTX-activated Na⁺ Channels**

The effects of naturally occurring (−)-cocaine in BTX-activated Na⁺ channels have been previously described (Wang, 1988). (−)-Cocaine induces long-lasting closures of muscle BTX-activated Na⁺ channels as shown in Fig. 2. Because BTX-activated Na⁺ channels open >97% of the time at voltage > −50 mV (Fig. 2, control; also Moczydlowski et al., 1984), the kinetics of the induced block can be conveniently characterized. The closed time distribution is well fitted by a single exponential with a closed time constant (τc) of 413 ms at +50 mV in this bilayer. The τc value is defined as mean LA dwell time in this report. Similarly, the open time distribution can be fitted with a single exponential and yields an open time constant (τo) of 263 ms. These time constants can be further related to the rate constants and the equilibrium dissociation constant (KD) as follows (for details, see Wang, 1988):

\[
k_{on} = 1/(\tau_o [L]) ;
\]

\[
k_{off} = 1/\tau_c ;
\]

\[
K_D = k_{off}/k_{on}.
\]
Control at +50 mV

300 μM (-) cocaine

10 mM ecgonine methyl ester

300 μM (-) norcocaine

1.2 mM (+) pseudococaine

Figure 2. Structure–activity relationship of cocaine homologues. Current records of single muscle BTX-activated Na⁺ channels at +50 mV is shown in the absence (control) and in the presence of 300 μM (-) cocaine, 10 mM ecgonine methyl ester, 300 μM (-) norcocaine, and 1.2 mM (+) pseudococaine. Only a 10-s record for each condition is displayed. The open and closed times were measured using Fetchan programs, and the time constants are listed in Table I. The cutoff duration for (-) cocaine induced closed time was 30 ms so that closed events of <30 ms were not counted (see Wang, 1988). No cutoff duration was implemented for (-) norcocaine and (+) pseudococaine. All drugs were applied internally and stirred vigorously for at least 30 s. The solid line drawn through the entire current traces indicates the zero current baseline. Records with discrete closing events, were purposely selected for baseline determination. The long closing events in the record of (-) norcocaine were rare events which were also present in the control. All current records were filtered at 100 Hz.

For cocaine, the calculated $k_{on}$, $k_{off}$, and $K_D$ values are listed in Table I. These kinetic data are useful for the comparison of the structure–activity relationship among various LA drugs.

Several cocaine homologues were applied in bilayers in order to examine their blocking effects on muscle BTX-activated Na⁺ channels. All cocaine homologues we surveyed appeared to be less potent than (-) cocaine in bilayers. For example,
removal of the O-benzoyl moiety from (-)cocaine yields a derivative called ecgonine methylester. This derivative essentially loses all the blocking activity even when applied internally at 10 mM concentration (Fig. 2). The lack of activity for ecgonine methylester is not surprising since most LA drugs require an aromatic group in their structure.

Another (-)cocaine homologue, (-)norcocaine, is a derivative after the removal of the methyl groups from the tertiary amine of (-)cocaine. (-)Norcocaine also produces channel closures in bilayers but the induced closures appear to be short-lived (Fig. 2). Although brief closures are also found in control records, they occur only infrequently (Moczydlowski et al., 1984). No attempts were made to correct these intrinsic events in our analysis. Both the open and closed time distributions could be fitted with single exponentials (Fig. 3) and the apparent $\tau_c$ and $\tau_o$ were estimated to be 21 and 52 ms, respectively. Because of relatively poor time resolution of bilayer system, these values should be considered as crude estimates. The estimated apparent $K_D$ value for (-)norcocaine in this bilayer is $-0.74$ mM, or $\sim 10$-fold larger than (-)cocaine (Table 1).

Moving the carbomethoxy group located at the tropone ring of (-)cocaine from an axial to an equatorial position yields a derivative called (+)pseudococaine. The potency of (+)pseudococaine is also much less than that of (-)cocaine (Fig. 2). Table I shows the estimated apparent $k_{on}$, $k_{off}$, and $K_D$ value for (+)pseudococaine. The major factor that governs the potency of these drugs appears to be the apparent $k_{off}$ value. For example, the apparent $k_{off}$ values of (-)cocaine and (-)norcocaine differ

### Table 1

<table>
<thead>
<tr>
<th>LA drug</th>
<th>Dissociation rate $k_{off}$ ($s^{-1}$)</th>
<th>Association rate $k_{on}$ ($M^{-1}s^{-1}$)</th>
<th>Equilibrium constant $K_D$ (mM)</th>
<th>No. of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) Cocaine</td>
<td>2.4</td>
<td>3.8</td>
<td>0.063</td>
<td>503</td>
</tr>
<tr>
<td>(-) Norcocaine</td>
<td>46.3 ± 5.7</td>
<td>7.12 ± 1.22</td>
<td>0.67 ± 0.15, n = 5</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>(+) Pseudococaine</td>
<td>33.3 ± 5.0</td>
<td>1.38 ± 0.38</td>
<td>2.53 ± 0.56, n = 4</td>
<td>&gt;4,000</td>
</tr>
<tr>
<td>(+) Cocaine</td>
<td>NA</td>
<td>NA</td>
<td>4.03 ± 1.19, n = 4</td>
<td>NA</td>
</tr>
<tr>
<td>(-) Pseudococaine</td>
<td>74.6 ± 20.6</td>
<td>0.98 ± 0.19</td>
<td>7.74 ± 1.92, n = 4</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Ecgonine methyl ester</td>
<td>NA</td>
<td>NA</td>
<td>&gt;10, n = 4</td>
<td>NA</td>
</tr>
</tbody>
</table>

Apparent rate and equilibrium constants were estimated by using Eqs. 1-4. The number of events for determining $\tau_c$ is listed. Kinetic data were presented without correction for missing events. More than three bilayers were used to determine the apparent kinetic constants for each drug. All drugs were applied internally at 0.3 mM for (-)cocaine and (-)norcocaine, 1.2 mM for (+) and (-)pseudococaine, 2-5 mM for (+)cocaine, and 10 mM for ecgonine methyl ester. NA, not available (i.e., the kinetic value cannot be obtained experimentally). *Averaged $K_D$ value and standard deviation taken from Wang (1988). †Data acquired at 400 Hz.
by ~20-fold whereas the apparent \( k_{on} \) values differ only by ~2-fold. Since different functional moieties on the \((-)\)-cocaine molecule are critical for its activity, it is likely that "multiple point" interactions occur between the \((-)\)-cocaine molecule and its binding site (Albert, 1973). For this to be true, the binding site should be able to differentiate the optical isomer of cocaine with relatively high stereoselectivity.

**Stereospecificity of Cocaine and Pseudococaine**

Synthetic stereoisomers of \((+)\)-cocaine and \((-)\)-pseudococaine were used to test the notion that multiple interactions were present between cocaine and its binding site. Fig. 4 shows that both \((+)\)-cocaine and \((-)\)-pseudococaine are less potent than their optical counterparts. Kinetic analyses of \((-)\)-pseudococaine show that this drug has a very fast apparent off-rate, \(~60/s\), or ~25 times faster than \((-)\)-cocaine whereas the apparent on-rate, \(~1.0/s) per \(10^{-4}\) M, is about four times slower than \((-)\)-cocaine (Table I). In contrast, \((+)\)-cocaine does not induce discrete closures in rabbit muscle BTX-activated \(Na^+\) channel but instead reduces the current amplitude and increases the open-channel noise. These effects are quite similar to those of a quaternary LA drug, QX-314. Like QX-314 and \((-)\)-cocaine, \((+)\)-cocaine also exhibits voltage-dependent binding interactions with the binding site. Fig. 5 shows that \((+)\)-cocaine reduces more \(Na^+\) current at \(+50\) mV than at \(-50\) mV. The \(K_D\) values for these drugs at a given voltage can be obtained by the following equation:

\[
K_D = \frac{[L] \cdot i_o}{i_o - i_b}
\] (4)
where \([L]\) is the drug concentration, \(i_0\) is the current amplitude without blocker, and \(i_b\) is the averaged current amplitude with an internal blocker. The voltage-dependent binding phenomenon will be described in more detail later (see Fig. 12). The \(K_D\) value for (+)cocaine at +50 mV was estimated to be 4.03 mM (Table I). In fact, the dose–response curves of (+)cocaine show that the data can be fitted by the Langmuir isotherm with a \(K_D\) of 4 mM (Fig. 6). As for comparison, the dose–response curve of (–)cocaine can also be described by the Langmuir isotherm with a \(K_D\) of 80 µM (Fig. 6; also see Wang, 1988), suggesting that one cocaine molecule closes one Na⁺ channel.

\[
\begin{align*}
3 \text{ mM} & \text{ (-) cocaine at } +50 \text{ mV} \\
3 \text{ mM} & \text{ (+) cocaine} \\
1.2 \text{ mM} & \text{ (+) pseudococaine} \\
1.2 \text{ mM} & \text{ (-) pseudococaine}
\end{align*}
\]

**Figure 4.** Stereoselectivity of cocaine and pseudococaine in single muscle BTX-activated Na⁺ channels. Current traces with 3 mM (-) and (+)cocaine were recorded at +50 mV. Long-lasting closures were readily detected in the (-)cocaine-treated channel but rarely in the (+)cocaine treated. The closures in the (+)cocaine record are rare events but are chosen here for baseline determination. Few long discrete closures were detected in the (+) and (-)pseudococaine-treated Na⁺ channel. All drugs were applied internally. The solid line indicates the zero current level.

The stereoselectivities for cocaine and pseudococaine are therefore about 50-fold and 3-fold, respectively. Such a high degree of stereoselectivity for cocaine optical isomers can be best explained by multiple interactions between cocaine and its receptor. For (±)pseudococaine, the interactions are weaker perhaps because of steric hindrance of carbomethoxy group, and hence yields less stereoselectivity.

**Cocaine Stereoselectivity in Sodium Channel Subtypes**

Na⁺ channels from different tissues are known to have different pharmacological properties in bilayers for TTX and μ-conotoxin. For example, heart Na⁺ channels
are relatively insensitive to TTX as compared to brain and muscle Na⁺ channels (Guo et al., 1987). µ-Conotoxin, on the other hand, blocks muscle Na⁺ channels at 50 nM concentration but not the brain and heart counterparts (Moczydlowski et al., 1986). To see whether these subtypes of Na⁺ channels have different sensitivity to the LA drugs, we have applied the cocaine isomers as probes.

Our results indicate that the relative stereoselectivity of the rabbit muscle, brain, and calf ventricular BTX-activated Na⁺ channels for (−) and (+) cocaine is the same in bilayers. Fig. 7 shows that both brain and ventricular BTX-activated Na⁺ channels are highly sensitive to (−)cocaine; long-lasting block can be readily detected. The $K_D$ value estimated for calf ventricular Na⁺ channels is about 20 µM which is about three to four times smaller than that for the brain Na⁺ channels (estimated to be ~80 µM) and the rabbit muscle counterparts (also ~80 µM). In contrast, (+) cocaine does not elicit long-lived closures in rabbit brain and calf heart Na⁺ channels; instead, it reduces the current amplitude and increases the open channel noise. The $K_D$ values calculated from the reduction of averaged Na⁺ current amplitude at +50 mV are 1.5 and 1.8 mM for rabbit brain and calf ventricular Na⁺ channels, respectively. The degree of the stereoselectivity of cocaine can be determined by the ratio of $K_D(+)_{cocaine}/K_D(−)_{cocaine}$. We found that the stereoselectivity ratio is approximately 50, 20, and 90 for rabbit muscle, brain, and calf ventricular Na⁺ channel subtypes, respectively. Because of this comparable stereoselectivity of (−)cocaine vs. (+)cocaine, we suggest that the structure of LA binding site in BTX-activated Na⁺ channel subtypes is relatively conserved.

**Stereoselectivity of Bupivacaine and Mepivacaine**

Like cocaine and pseudococaine, bupivacaine (R = −C₄H₉) and mepivacaine (R = −CH₃) also contain chiral carbons. The chiral carbon is adjacent to the tertiary amine. To see whether other clinically used LA drugs also exhibit similar stereoselectivity we chose these two drugs as probes. We observed that (S,−)bupivacaine and (S,+)mepivacaine are more potent than (R,+)bupivacaine and (R,−)mepivacaine,
respectively, in blocking muscle BTX-activated Na⁺ currents. Fig. 8 shows that (-)bupivacaine induces discrete closed events with a mean closed time of 110 ms. In contrast, (+)bupivacaine, (+)mepivacaine, and (-)mepivacaine reduce current amplitude and increase the open-channel noise. The $K_D$ values at +50 mV for these drugs were estimated to be 0.47, 2.8, 1.2, and 3.7 mM for (-)bupivacaine, (+)bupivacaine, (+)mepivacaine, and (-)mepivacaine, respectively. The dose–response curves of (-) and (+)bupivacaine at +50 mV also yield similar $K_D$ values at 0.40 and 2.3 mM, respectively (Fig. 6). The stereoselectivity ratios for bupivacaine and mepivacaine were calculated to be about sixfold and threefold, respectively. We conclude therefore that the S form bupivacaine homologues are more potent than R form in bilayers. This conclusion is further supported by the results of (S, )ropivacaine (R = -C₃H₇) and its stereoisomer. Again, the S form of this drug is more potent than the R form (data not shown). Our results thus clearly demonstrate that the LA receptor is also stereoselective for optical isomers of bupivacaine homologues.

**Structure–Activity Relationship of Bupivacaine Homologues**

Since (S, ) bupivacaine appears to be much more potent than (S, +) mepivacaine (Fig. 8), it is possible that the longer the carbon number of the alkyl group attached

![Figure 6. Dose-response curves for various LA drugs. The fractional open times ($f_o$) of (-)caine and (-)bupivacaine at +50 mV are plotted against drug concentration. The solid line is drawn according to the Langmuir adsorption isotherm: $f_o = [K_D]/(K_D + [L])$, where $f_o$ is the ratio of the time in the open state to the duration of the record, [L] is the drug concentration, $K_D$ is the drug concentration at which $f_o = 0.5$. For (+)caine and (+)bupivacaine, values of $i_o/i_0$, as described in Eq. 4 are plotted against drug concentration. These data can also be fitted with the Langmuir isotherm: $i_o/i_0 = K_D/[K_D + [L]]$ as drawn in solid lines. The $K_D$ values obtained from dose–response curve are 80 μM, 400 μM, 2.3 mM, and 4 mM for (-)caine, (-)bupivacaine, (+)bupivacaine, and (+)caine, respectively. One to four bilayers were performed for each drug.](https://example.com/figure6.png)
to the tertiary amine group, the longer the drug dwell time. The bupivacaine homologues of N-substitution are available in their racemic mixtures or individual isomers. Among them are N-substitutions of N-propyl (R = C₃H₇), N-butyl (R = C₄H₉), N-pentyl (R = C₅H₁₁), and N-octyl (R = C₈H₁₇). The induced block on Na⁺ channels by these homologues differs significantly. In fact, the longer the carbon number, the longer the closed time was found as shown in the current records (Fig. 9). Unlike mepivacaine the on- and off-rate constants of these homologues could also be measured (Fig. 10). Because ropivacaine induced relative short closures (τₜ = 37.2 ± 6.5 ms, n = 7, at 100 Hz sampling rate), we also

measured closing times at 400 Hz sampling rate which yielded a lower value (τₜ = 28.7 ± 8.5 ms, n = 6). Both methods yielded comparable Kᵦ values (1.06 ± 0.28 vs. 1.20 ± 0.11 mM, respectively). Further reduction of the bilayer area is required for an accurate measurement of this fast-blocking drug. Whereas the S-form optical isomers for ropivacaine and bupivacaine were used for Figs. 9 and 10, only racemic mixtures of R = C₈H₁₇ and R = C₈H₁₇ were available. Fortunately, since the R-form isomers of bupivacaine homologues have a potency around millimolar range (Fig. 8), the induced blocks by R = C₈H₁₁ and R = C₈H₁₇ racemic mixtures at the submillimolar range are likely to be elicited by the S-form
isomer. Accordingly, all the $k_{on}$ values were estimated with the assumed S-form concentration, i.e., half of the total concentration applied.

According to our kinetic data, the potency of these homologues appear to be highly dependent on the dwell time of the LA drugs. We found that the higher the carbon number, the longer the LA dwell time and the stronger the binding affinity (Fig. 10). From the estimated $K_D$ value, it is possible to calculate the free energy of binding, $\Delta G^o$ eq:

$$\Delta G^o_{eq} = RT (\ln K_D)$$

300 $\mu$M (S,−) bupivacaine

1.2 mM (R,+) bupivacaine

1.5 mM (S,+) mepivacaine

1.5 mM (R,−) mepivacaine

**Figure 8.** Stereoselectivity of bupivacaine and mepivacaine in rabbit muscle BTX-activated Na+ channels. Current traces were taken from bilayers treated with (−) and (+)bupivacaine or (+) and (−)mepivacaine at the indicated concentration. The $\tau_o$ and $\tau_e$ constants for (−)bupivacaine in this bilayer are 173 and 110 ms, respectively. Kinetic data cannot be accurately obtained from (+)bupivacaine or (−) and (+) mepivacaine. Drugs were applied internally to the Na+ channel. The solid line indicates the zero current level. Records of mepivacaine with conspicuous closed events were selected for baseline determination. Such events may be due to spontaneous channel closures. Currents were filtered at 100 Hz.

Our data show that for each additional $-\text{CH}_2$ group in the N-substitution, ~450 cal/mol energy is released upon binding. This result strongly suggests that hydrophobic interactions are involved in the binding between the N-substitution moiety of bupivacaine homologues and the LA binding site. Since direct kinetic analyses on mepivacaine are not feasible at the present time, we could not include R = $-\text{CH}_3$ in Fig. 10. Interestingly, however, the $K_D$ value (1.2 mM) calculated by Eq. 4 does not appear to fall in line with other bupivacaine homologues. Whether this deviation is due to largely the on-rate or off-rate value remains to be investigated.
A Common Binding Site for Cocaine and Bupivacaine Optical Isomers

Although our results on cocaine and bupivacaine stereoselectivity support the notion of multiple interactions between the receptor and LA drug, it can still be argued that the optical isomers bind to two separate sites and elicit two different blocking effects (Strichartz and Ritchie, 1987). To rule out this possibility, direct competition experiments were performed in bilayers.

Because the blocking effect of (+)cocaine and (+)bupivacaine is very different from (-)cocaine, it is possible to study their competitive binding when drugs are added consecutively. Fig. 11 shows that (+)cocaine appears to compete directly with

\[ R = -C_3H_7, \text{(+)ropivacaine, 1.0 mM} \]

\[ R = -C_4H_9, \text{(-)bupivacaine, 300 \mu M} \]

\[ R = -C_5H_{11}, \text{(+)RAD 393, 300 \mu M} \]

\[ R = -C_8H_{17}, \text{(+)1-octyl-2',6'-pipecoloxylidide, 100 \mu M} \]

**Figure 9.** The effects of bupivacaine homologues on muscle BTX-activated Na⁺ channels. Current traces at the indicated concentration of various bupivacaine homologues are displayed. Notice that the drug dwell time increases as the carbon number in R group increases. Drugs were applied internally to the Na⁺ channel. Assuming that the S form induces the long-lasting block for the (±) RAD-393 and (±) 1-octyl-2',6'-pipecoloxylidide, the actual concentration responsive for the blocking effect may be half of the indicated value. The solid line indicates the zero current level. Currents were filtered at 100 Hz.

the (-)cocaine binding site in rabbit muscle BTX-activated Na⁺ channels. The bilayer was first treated with 300 \( \mu M \) (-)cocaine externally. The on- and off-rate kinetics were determined by the measurements of \( \tau_o \) and \( \tau_c \). The estimated \( K_D \) yielded a value of 115 \( \mu M \), which was slightly higher than the obtained \( K_D \) value when (-)cocaine was applied internally (Wang, 1988). After the measurement, additional 4 mM (+)cocaine was further applied to the external side. The Na⁺ current amplitude was clearly reduced. In addition, the on-rate constant was found to be reduced by \~50\% whereas the off-rate constant was little changed. Similar effects were obtained for internal (-)cocaine and internal (+)bupivacaine drugs (Fig. 11).
FIGURE 10. Kinetic constants of the induced closures in muscle BTX-activated Na⁺ channels by bupivacaine homologues. (A) The rate constants were determined from the current traces of a 5-min record using Eqs. 1 and 2. (○) \( k_{on} \) values are shown with a dashed line; (●) \( k_{off} \) values are shown with a solid line. Lines were drawn by eye. (B) Equilibrium dissociation constants \( K_D \) were calculated according to Eq. 3 using the data shown in A plus some additional data, and plotted against the carbon number of the R substitution on the tertiary amine. The line was the least square fit of the data, \( \log K_D = 0.97 - 0.32 \times (\text{carbon number}) \), where \( K_D \) unit is in millimolar. Data were filtered at 100 Hz and sampled at 100 Hz except for ropivacaine, which was sampled at 400 Hz.

The on-rate of (−)-cocaine was again slowed by about twofold in the presence of 2 mM (+)-bupivacaine, whereas the off-rate was little changed. These results are essentially identical to those found previously in the QX-314 and (−)-cocaine competition experiments (Wang, 1988) and can be described by the following scheme:

\[
\begin{align*}
\text{(-)-cocaine} & \quad \text{O} \quad \text{O \cdot (-)-cocaine (blocked)} \\
\text{(+)-cocaine} & \quad \text{O} \quad \text{O \cdot (+)-cocaine (flickering)}
\end{align*}
\]

Because the two drugs bind to the same site, the (−)-cocaine molecule will have to wait for the unoccupied receptor when (+)-cocaine is present at a relative high concentration. Consequently, the apparent on-rate constant for (−)-cocaine is reduced \( (1.49 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \text{ vs } 0.77 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}) \). According to the above scheme, the \( K_D \) value of (+)-cocaine can also be estimated as follows:

\[
\tau_{o(\text{obs})} = \tau_o \left(1 + \frac{[\text{(+)-cocaine}]}{K_D}\right),
\]

(6)
where $\tau_{\text{glob}}$ and $\tau_0$ are the mean open times with both (+) and (-)cocaine present and (-)cocaine alone, respectively. The calculated $K_D$ value for (+)cocaine is 4.25 mM, which is in good agreement with the data from dose-response curves (4 mM in Fig. 6). Furthermore, the unbinding of (-)cocaine should not be affected by the presence of (+)cocaine if the two drugs compete for the same site (1.72 vs. 1.93 s⁻¹). In agreement with this notion, a direct competition experiment between (-)bupivacaine and (+)bupivacaine also yielded the same conclusion (data not shown). Our results thus support that a common binding site exists for both cocaine and bupivacaine isomers.

**Voltage-dependent Binding of Cocaine and Bupivacaine Homologues**

We have previously shown that (-)cocaine, (-)bupivacaine, and QX-314 exhibit a strong voltage-dependent binding phenomenon with an estimated equivalent valence

\[
\text{Figure 11. Direct competition among (--)cocaine, (+)cocaine, and (+)bupivacaine for a common LA binding site in muscle BTX-activated Na⁺ channels. (A) Current traces with 300 \mu M external (--)cocaine at +50 mV were recorded first for 5 min. (B) Additional 4 mM (+)cocaine was then added externally to the Na⁺ channel. The open duration was found to be lengthened by (+)cocaine whereas the closed duration was little changed. Kinetic analyses of current records for A yielded $K_{on} = 1.49 \times 10^4$ M⁻¹s⁻¹ and $k_{off} = 1.72$ s⁻¹, whereas for B, $k_{on} = 7.68 \times 10^5$ M⁻¹s⁻¹ and $k_{off} = 1.93$ s⁻¹. (C) Current traces with 300 \mu M internal (--)cocaine at +50 mV were recorded. (D) Additional 2 mM (+)bupivacaine was then added internally to the Na⁺ channel. Notice that the open duration was again lengthened by (+)bupivacaine but not the closed duration. Kinetic data for C were $k_{on} = 3.33 \times 10^4$ M⁻¹s⁻¹ and $k_{off} = 2.3$ s⁻¹, whereas for D, $k_{on} = 1.71 \times 10^4$ M⁻¹s⁻¹ and $k_{off} = 2.0$ s⁻¹. The calculated $K_D$ according to Eq. 5 yielded a value of 2.11 mM for (+)bupivacaine which is similar to 2.3 mM obtained in Fig. 6.}
(δ) of 0.55 ± 0.07, 0.58, and 0.48 ± 0.02, respectively (Wang, 1988). To test whether the voltage-dependent binding phenomenon is similar among cocaine and bupivacaine homologues we compared \( K_D \) values of (+)cocaine, (-)ropivacaine, (+)l-octyl-2',6'-picecoloxylidide, and (-)cocaine under various voltage conditions. Fig. 12 shows that all drugs tested have a higher binding affinity when the membrane is more depolarized. The estimated equivalent valence, \( \delta \), falls within a range between 0.4 and 0.58. All other drugs used in this report, without exception, exhibit a blocking effect stronger at +50 mV than at -50 mV (data not shown). This result demonstrates that the voltage dependent binding is a common phenomenon for cocaine and bupivacaine homologues.

**Changes of LA Stereoselectivity by BTX?**

During the course of our bilayer studies we have come to realize that the stereoselectivity for bupivacaine and cocaine isomers in bilayers is drastically altered by BTX as compared to that in intact tissues. Unlike in bilayers, (+)bupivacaine was found to be about two-fold more potent than (-)bupivacaine in the node of Ranvier D. Chernoff, personal communication). Similarly, (+)cocaine and (-)cocaine were found to be about equally potent at the mM range in blocking the excitability of frog sciatic nerve (Gottlieb, 1923; Albert, 1973). To substantiate that the change of LA stereoselectivity by BTX is also a common phenomenon, we have selected the RAC stereoisomers for further investigation. To our knowledge, RAC series are the most.
characterized LA stereoisomers in intact tissues (Hille et al., 1975; Yeh, 1980). Fig. 13 shows that in bilayers internal quaternary and tertiary LA, RAC421 (II) and RAC109 (II), are more potent than the stereoisomers of RAC421 (I) and RAC109 (I), respectively, in blocking muscle BTX-activated Na⁺ channels. External RAC421 (II) or (I) has no effects on BTX-activated Na⁺ channel because these compounds contain a permanent charge which can not pass the membrane barrier. The potency difference for internal RAC421 (I,II) and RAC109 (I,II) stereoisomers is estimated to be about threefold, respectively. In two separate experiments we have also found that brain BTX-activated Na⁺ channels exhibit the same stereoselectivity toward RAC compounds (data not shown), suggesting again that the stereoselectivity is conserved in the Na⁺ channel subtypes.

In the absence of BTX, however, internal RAC421 (I) was found to be 10-fold more effective in producing block at 0 mV than RAC421 (II) isomer in squid axons (Yeh, 1980). Clearly, the order of potency is reversed in bilayers by BTX. The same inversion of order was found for the tertiary amine RAC109 (Fig. 10 vs. Fig. 4 in Yeh, 1980; also Hille et al., 1975). All of these results together suggest that the change of LA stereoselectivity by BTX is also a common phenomenon.

**Figure 13.** Effects of RAC stereoisomers on muscle BTX-activated Na⁺ channels. Na⁺ current traces were recorded at +50 mV after the bilayers were treated with internal RAC compound at the indicated concentration. The kinetic analyses for RAC421 (II) and RAC109 (II) were barely possible with \( k_{on} = 2.89 \times 10^4 \text{M}^{-1}\text{s}^{-1} \), \( k_{off} = 53.5 \text{s}^{-1} \), and \( K_D = 1.85 \text{mM} \) for RAC421 (II), and \( k_{on} = 1.67 \times 10^4 \text{M}^{-1}\text{s}^{-1} \), \( k_{off} = 38.9 \text{s}^{-1} \), and \( K_D = 2.3 \text{mM} \) for RAC109 (II). \( K_D \) values for RAC421 (I) and RAC109 (I) were calculated using Eq. 4, which yielded a value of 5.7 and 6.6 mM, respectively. The solid line indicates the zero current level. Currents were filtered at 100 Hz.
It is noteworthy that RAC 109 (I) was found about five- to sevenfold more potent than RAC109 (II) in the inhibition of the equilibrium binding of \[^3H\]batrachotoxin-A-20-\(\alpha\)-benzoate (BTX-B) using rat brain synaptosomal preparation (Postma and Catterall, 1984) or using rat cardiac myocytes (Hill et al., 1988). In such an assay system, however, RAC compounds are believed to bind first with the inactivated normal Na\(^+\) channels and upon binding inhibits the BTX binding at equilibrium. Consequently, the result of RAC109 stereoselectivity determined by the inhibition of \[^3H\]BTX-B binding should not be considered as inconsistent with our findings reported here.

**DISCUSSION**

Tertiary and quaternary amine LA drugs are small chemicals with a molecular mass ~300 D. Two functional groups are invariably present in their structure (Ritchie and Greene, 1985; Strichartz and Ritchie, 1987): a hydrophobic aromatic ring and a tertiary (or quaternary) amine separated by an intermediate alkyl chain. In general, the aromatic ring and the intermediate alkyl chain are linked by either an amide bond (such as bupivacaine homologues) or by an ester bond (such as cocaine homologues). Almost all chemicals with such basic structure have LA activity, but with different potency. Our experiments were designed to study the structural determinants that may govern the dwell time and the binding affinity of various LA drugs at the single channel level. Based on the results from dose-response curves, single exponential distributions of LA dwell times, and direct competition experiments, we suggest that for these LA drugs there is only one binding site per Na\(^+\) channel in the bilayer system.

*Structural Determinants of Cocaine*

Cocaine, being the first clinically used local anesthetic, has been a prototype for LA drug synthesis. The chemical structure of the cocaine molecule is shown in the following scheme:

![Cocaine and Tropane](image)

It contains a tropane ring which is extremely rigid as compared to other LA drugs. Synthetic LA drugs normally substitute the tropane ring with an alkyl chain which can rotate freely between the \(-\text{c-c-}\) bond.

Our results suggest that cocaine binds to its binding site involving several parts of its molecule. Just a slight change in the cocaine structure can drastically alter its binding characteristics. Removal of a simple methyl group from the tertiary amine...
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greatly reduces the mean dwell time of the cocaine molecule from 400 to <25 ms. Since an additional methyl group may enhance the regional hydrophobic interaction, the implication of this result is that the tertiary amine is actually located near or at the hydrophobic region in the LA-receptor complex. Further supporting evidence will be provided for this notion using bupivacaine homologues.

The tropane ring of cocaine or the substituted intermediate alkyl chain of synthetic LA drugs may also involve in LA-receptor binding interactions since the position of the carbomethoxy group of cocaine appears to be critical for the cocaine dwell time. Moving the carbomethoxy group from axial to equatorial position greatly reduces the cocaine dwell time. This particular substitution in the tropane ring may perturb the interaction between the intermediate alkyl chain of LA drug and the receptor. Such a perturbation may arise from steric hinderance or disruption of hydrophobic interactions between the intermediate alkyl chain and the receptor.

The most important structural determinant for cocaine-induced closures appears to be the hydrophobic aromatic ring. Removal of this ring by breaking the O-benzoyl bond completely destroys the cocaine activity. The derivative, ecgonine methylester, contains a tropane structure but this drug does not induce channel closures. Could the aromatic ring involve in the binding interactions? This possibility is likely since ecgonine methylester does not inhibit effectively the equilibrium binding of [3H]BTX-B as compared to other cocaine homologues (Reith et al., 1986), suggesting that the aromatic ring is required for binding. One interesting speculation is that the aromatic ring of LA drugs not only interacts with a hydrophobic region of the conduction pathway of the Na⁺ channel but also at the same time blocks the channel by plugging the pore. Such a scenario was proposed as a mechanism for LA action in the acetylcholine receptor using site-directed mutagenesis approach (for details, see Leonard et al., 1988; Miller, 1989). A similar approach may be applied to deduce the LA binding site of the Na⁺ channel in the future.

Hydrophobic Interactions Determine the LA Dwell Time

Our results from cocaine homologues support the notion that there are two hydrophobic binding domains: one is near the tertiary amine (norcocaine vs cocaine) and the other includes the hydrophobic aromatic ring (ecgonine methylester vs. cocaine). Less known so far is the hydrophobic binding region near the tertiary amine. Since the tertiary amine can be protonated at neutral pH, the amine region is traditionally thought to be the hydrophilic portion of LA drugs (Ritchie and Greene, 1985). However, the tertiary amine can also be in neutral form, which can readily penetrate the membrane (Narahashi et al., 1970). The LA drugs in its neutral form can, therefore, be easily embedded in a hydrophobic region of the Na⁺ channel. To test whether the tertiary amine of LA drugs is located at or near a hydrophobic binding domain of LA receptor we chose to study the bupivacaine homologues.

From the mean dwell time of bupivacaine homologues in bilayers, it is clear that hydrophobic interactions occur near or at the tertiary amine of LA drugs and play an important role in LA binding. Different binding affinities of various bupivacaine homologues are largely determined by the drug dwell time (Fig. 8). In general, the higher the carbon number attached to the tertiary amine the longer the LA mean dwell time and the higher the binding affinity. Except for mepivacaine, the free
energy involved in each carbon addition of bupivacaine homologues is calculated to be \( \sim 450 \text{ cal/mol} \). This estimated value is comparable to the hydrophobic interactions of tetraethylammonium derivative with K\(^+\) channels (Yeh, 1982). In that series, each carbon length contributes \( \sim 600 \text{ cal/mol} \). The theoretical maximal value is \( \sim 1,000 \text{ cal/mol} \) for removing a hydrophobic carbon (-CH\(_2\)) from an aqueous solution to a hydrophobic environment (Kauzman, 1959). Our results thus suggest that the LA receptor site may be in a relatively hydrophobic environment.

**Stereoselectivity of LA Receptor for Cocaine and Bupivacaine Isomers**

Stereochemical information can provide important pieces of evidence for specific topology of the Na\(^+\) channel-LA complex (Hille et al., 1975; Yeh, 1980). If there are multiple contacts during binding interactions, the receptor must be able to differentiate different parts of the drug. This stereoselectivity has to be also based on the specific configuration of the receptor site (Fersht, 1985).

Our results clearly show that the LA binding site can be highly stereoselective. As a rule, the R-form configuration of bupivacaine homologues has a \( K_D \) value at the mM range. The other configuration (S form) of bupivacaine homologues is always more potent than the R form. Similarly, in cocaine homologues a consistent stereoselectivity is also observed. Both \((-)\) cocaine and \((+)\) pseudococaine are found more potent than their counterparts. From dose–response curves and direct competition experiments between cocaine and bupivacaine isomers, we conclude that there is only one single LA binding site which interacts with \((+/−)\) cocaine and \((+/−)\) bupivacaine isomers. The low affinity and the short dwell time of \((+)\) cocaine and \((+)\) bupivacaine in the Na\(^+\) channel are probably due to their inability to form proper hydrophobic interactions between local anesthetics and the receptor site.

Using stereoisomers as probes for the subtypes of Na\(^+\) channels we further found that Na\(^+\) channels from muscle, brain, and ventricular tissues display a similar stereoselectivity toward cocaine isomers. Again, \((-)\) cocaine is \( \geq 20 \)-fold more potent than \((+)\) cocaine for all subtypes. This result is expected if the configuration of the LA receptors for the Na\(^+\) channel subtypes are very similar. In other words, the structure of the LA binding site must be relatively conserved for muscle, brain, and ventricular Na\(^+\) channels. This structural conservation of the LA binding site is distinctly different from the saxitoxin and \(\mu\)-conotoxin receptor in the Na\(^+\) channel subtypes (Moczydlowski et al., 1986a). Whether structural conservation of the LA binding site is due to functional importance of this region remains to be explored.

**Possible Explanations for the Change of LA Stereoselectivity by BTX**

It was rather unexpected that a drastically different stereoselectivity exists in bilayers for bupivacaine, cocaine, and RAC compounds as compared to the previously reported stereoselectivity in the intact tissues. In nerve fibers the RAC109 (I) and RAC421 (I) are found more potent than RAC109 (II) and RAC421 (II), respectively (Hille et al., 1975; Yeh, 1980), whereas in bilayers the reverse is true (Fig. 13). The reason for this altered stereoselectivity in bilayers is unclear. Two possible explanations will be considered here. First, it is conceivable that there is an additional LA binding site in intact tissues (e.g., Mrose and Ritchie, 1978; Strichartz and Ritchie, 1987) which has a different stereoselectivity toward cocaine, bupivacaine, and RAC
isomers. In theory, BTX can modify one particular binding site so that it no longer binds with LA drugs in bilayers. As a result, the stereoselectivity in bilayers reflects only one of the two binding sites in native membranes.

Alternatively, a modulated receptor hypothesis for LA binding originally proposed by Hille (1977) and others (Khodorov, 1976; Hondeghem and Katzung, 1977) may also explain these results. The modulated receptor hypothesis states that (a) the LA receptor has a voltage- and time-dependent conformation and (b) different conformations of Na⁺ channels bind with LA drugs with different affinities. For this hypothesis to be true, the LA binding site must be structurally rearranged as the channel changes its conformation from the resting to the open and then to the inactivated Na⁺ channel. Consequently, it is possible that the open and closed Na⁺ channels have a different binding affinity as well as a different stereoselectivity toward LA drugs. In bilayers, the BTX-activated Na⁺ channels open most of the time at voltages > −50 mV (Krueger et al., 1983; Moczydlowski et al., 1984). The stereoselectivity for LA isomers in bilayers, therefore, may correspond exclusively to the open channel–LA interactions (Wang, 1988), whereas in intact tissue, Na⁺ channels open briefly during depolarization, usually < 1 ms in duration (Aldrich et al., 1983). Even when the nerve is stimulated at 1 Hz, the duration of the open conformation corresponds to only ~0.1% of the total time. Since most of the time, the Na⁺ channel stays in its closed conformation, the stereoselectivity in intact nerve may reflect primarily the closed channel–LA interactions. The unique stereoselectivity of LA binding during channel opening will be difficult to detect because of the short open time. Whatever the explanation for the unusual LA stereoselectivity in bilayers, it is interesting to note here that previously BTX has also revealed a unique voltage-dependent binding of TTX (Krueger et al., 1983; Moczydlowski et al., 1984; Rando and Strichartz, 1986; Green et al., 1987) that is not observed in normal Na⁺ channels.

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