Altered Stereoselectivity of Cocaine and Bupivacaine Isomers in Normal and Batrachotoxin-modified Na⁺ Channels

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ABSTRACT The inhibitory effects of local anesthetics (LAs) of cocaine and bupivacaine optical isomers on Na⁺ currents were studied in clonal GH3 cells under whole-cell patch clamp conditions. At holding potential of -100 mV, all four isomers inhibited peak Na⁺ currents when the cell was stimulated infrequently. The dose–response curves of this tonic block of peak Na⁺ currents by (-)/(+) cocaine and (-)/(+) bupivacaine were well fitted by the Langmuir isotherm, suggesting that one LA isomer blocked one Na⁺ channel. Each pair of isomers showed no greater than a twofold difference in stereoselectivity toward Na⁺ channels. Additional block of Na⁺ currents occurred when the cell was stimulated at 2 Hz. This use-dependent block was also observed in all four isomers, which again displayed little stereoselectivity. The voltage dependence of the use-dependent block produced by cocaine isomers did not overlap with the activation of Na⁺ channels but did overlap with the steady-state inactivation (h∞), indicating that cocaine can bind directly to the inactivated state of Na⁺ channels before channel opening. In comparison, the peak batrachotoxin (BTX)-modified Na⁺ currents were little inhibited by cocaine and bupivacaine isomers. However, the maintained BTX-modified Na⁺ currents were highly sensitive toward the (-) form of cocaine and bupivacaine isomers during a prolonged depolarization. As a result, a profound time-dependent block of BTX-modified Na⁺ currents was evident in the presence of these LA isomers. The estimated values of the equilibrium dissociation constant (Kᵦ in micromolar) at +50 mV were 35.8, 661, 7.0, and 222 for (-)/(+) cocaine and (-)/(+) bupivacaine, respectively. Although chloramine-T (CT) also modified the fast inactivation of Na⁺ channels and gave rise to a maintained Na⁺ current during a prolonged depolarization, LA isomers showed no greater stereoselectivity in blocking this maintained current than in blocking the normal transient Na⁺ current. We conclude that (a) cocaine and bupivacaine isomers exhibit only weak stereoselectivity toward the LA receptor in normal and CT-treated Na⁺ channels, (b) BTX drastically modifies the configuration of the LA binding site so that the LA stereoselectivity of the open Na⁺ channels is altered by an order of magnitude, and (c) the (-) forms of cocaine and bupivacaine interact strongly with the open state of BTX-modified Na⁺ channels but...
only weakly, if at all, with the closed state. The last finding may explain why most LA drugs were reported to be less effective toward BTX-modified Na\(^+\) channels. Prolonged depolarization of membrane is required to observe the full effect of these LAs in BTX-modified Na\(^+\) channels.

**INTRODUCTION**

Local anesthetics (LAs) are drugs that reversibly block the propagation of action potentials in excitable membranes (Ritchie and Greene, 1985). Except for benzo- caine, clinically used LAs such as bupivacaine and cocaine consist of an aromatic ring, an intermediate amide (bupivacaine) or ester bond (cocaine), and a tertiary amine attached to alkyl groups (see diagram, with \(*\) denoting the asymmetrical carbon). All LA drugs can interact directly with the voltage-gated Na\(^+\) channel and, as a result, prohibit Na\(^+\) ions from passing through the channel.

The interactions between LAs and their Na\(^+\) channel binding site(s) are known to be extremely complicated (Hille, 1984). The complication is mainly due to the dynamic properties of the Na\(^+\) channel protein, which has many voltage-sensitive state transitions upon depolarization. To circumvent this complication, Moczydlofski, Uehara, Guo, and Heiny (1986) first adopted the planar bilayer system to study the blocking effect of LAs in batrachotoxin (BTX)-modified Na\(^+\) channels under equilibrium conditions. In an attempt to define the topology of this LA binding site, we have recently reported that a high LA stereoselectivity of cocaine and bupivacaine is present in BTX-modified channels (Wang, 1990), compared with the known weak or moderate LA stereoselectivity in unmodified Na\(^+\) channels (Gottlieb, 1923; Hille, Courtney, and Dum, 1975; Yeh, 1980). Direct comparison of stereoselectivity in BTX-modified and unmodified Na\(^+\) channels, however, was not possible in the previous study because the large capacitance of the conventional bilayer system did not permit us to study the transient open state of Na\(^+\) channels.

In this study we used the GH3 cell preparation to examine directly the LA stereoselectivity in BTX-modified and unmodified Na\(^+\) channels under whole-cell voltage clamp conditions. Our results show that in neuronal GH3 cells the LA stereoselectivity of BTX-modified Na\(^+\) channels is nearly the same as that of their brain counterparts in planar bilayers, thus demonstrating the validity of the bilayer study. Furthermore, we provide direct evidence that the stereoselectivity of cocaine
and bupivacaine is indeed altered in BTX-modified Na\(^+\) channels. Both bupivacaine and cocaine are shown to be open channel blockers in BTX-modified Na\(^+\) channels and to have little, if any, binding interactions with the closed, BTX-modified Na\(^+\) channels. In contrast, LAs do interact with the closed states of normal Na\(^+\) channels as indicated by the observed tonic block of Na\(^+\) currents and by the observed voltage dependence of the use-dependent block.

**MATERIALS AND METHODS**

**Chemicals**

(-)-Cocaine and (+)-cocaine were obtained from Dr. Rao Rapaka, National Institute of Drug Abuse, Bethesda, MD. (-)-Bupivacaine and (+)-bupivacaine were gifts from Dr. Rune Sandberg of Astra Pain Control, Sodertalje, Sweden. BTX was provided by Dr. John Daly, National Institutes of Health, Bethesda, MD. Tetrodotoxin (TTX) was purchased from Calbiochem Corp. (La Jolla, CA). Chloramine-T (CT) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

**Whole-Cell Voltage Clamp, Data Acquisition, and Analysis**

The whole-cell variant of the patch clamp method (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to measure Na\(^+\) currents in GH\(_3\) cells at room temperature (23 ± 2°C). GH\(_3\) cells were maintained as described by Cota and Armstrong (1989). Experiments were performed with the external solution containing (mM) 150 choline-Cl, 0.2 CdCl\(_2\), and 10 HEPES adjusted to pH 7.4 with TMA-OH (Cota and Armstrong, 1989), and the internal solution containing (mM) 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. BTX was applied intracellularly within the micropipette at a final concentration of 5 μM. Fast inactivating Na\(^+\) currents were converted to BTX-modified noninactivating Na\(^+\) currents by repetitive depolarizations as described previously (Wang and Wang, 1992). The holding potential was set at −100 mV in all experiments.

Creation of voltage clamp pulses, data acquisition, and data analysis were performed as described in Wang and Wang (1992). After CT treatment, leak currents generally increased significantly. As a result, no detailed study was performed in CT-treated cells. Weighted least-squares curve fitting was performed using the Levenberg-Marquardt algorithm (Marquardt, 1963). Results are expressed as mean ± SE.

**RESULTS**

**Effects of Cocaine and Bupivacaine Stereoisomers on Normal Na\(^+\) Currents in GH\(_3\) Cells**

Under reversed Na\(^+\) gradient conditions, outward Na\(^+\) currents in GH\(_3\) cells are visible at all voltages above −40 mV (Fig. 1A) as described previously (Cota and Armstrong, 1989; Wang and Wang, 1992). The peak Na\(^+\) current and voltage relationship is shown in Fig. 1C (open circles). Treatment of 200 μM (−)-cocaine reduces the Na\(^+\) current amplitude at various test voltages by ~50% (Fig. 1, B and C). There is no evidence for stronger inhibition of peak Na\(^+\) currents at more positive potentials. The Na\(^+\) current kinetics are little affected; both the rising and the declining phases of Na\(^+\) currents remain relatively fast and the current inactivation reaches its completion within 5 ms of the test pulse with a single exponential
time constant ($\tau_h$, at +30 mV) value of 0.55 ± 0.03 and 0.54 ± 0.04 ms (mean ± SE, n = 5) for control and (-)cocaine-treated cells, respectively. Similar results were found at all voltages. Thus, gating of Na$^+$ channels when not blocked at rest remains normal during depolarization.

The stereoselectivity of cocaine isomers in normal Na$^+$ channels is found to be minimal (i.e., no greater than twofold; Fig. 2). For example, (+)cocaine at 300 μM reduces the Na$^+$ currents (Fig. 2 B) in a manner similar to that of (-)cocaine at 200 μM (Fig. 2 A). Furthermore, there are no apparent changes in current kinetics by (+)cocaine. The degree of stereoselectivity of bupivacaine isomers in GH3 cells is also minimal as shown in Fig. 2, C and D; (+)bupivacaine at 100 μM is found to be almost as potent as 100 μM (-)bupivacaine. Again, bupivacaine isomers have little effect on Na$^+$ current kinetics. These results confirm previous reports (Hille et al., 1975; Yeh, 1980; Guo, Castle, Cehrnoff, and Strichartz, 1991) that normal Na$^+$ channels display only weak or moderate stereoselectivity toward LA optical isomers.

Use-dependent Block of Cocaine and Bupivacaine Stereoisomers

In addition to the inhibition of Na$^+$ currents by a single pulse in the presence of cocaine and bupivacaine, repetitive depolarizations of the membrane produce an additional block of Na$^+$ currents. Both (-) and (+)cocaine elicit such a use-dependent block, as shown in Fig. 2, A and B, by repetitive depolarizations of 30 mV
for 50 ms. A similar use-dependent block by (-) and (+)bupivacaine is found in GH3 cells (Fig. 2, C and D). The rate of the use-dependent block is relatively fast at the concentrations applied. Within 5–10 pulses the use-dependent block reaches its steady state. Our results thus clearly demonstrate that cocaine and bupivacaine stereoisomers produce both tonic and use-dependent block (Fig. 2) like many other LAs. Stereoisomers of these compounds, therefore, cannot be used as tools to differentiate the tonic block from the use-dependent block in GH3 cells. In contrast, Yeh (1980) reported that RAC stereoisomers are not stereoselective in the resting (tonic) block of Na+ currents in squid axons but are moderately stereoselective in the use-dependent block. Evidently, this is not the case in GH3 cells for cocaine and bupivacaine isomers.

![Figure 2](image-url)

**Figure 2.** The stereoselectivity of Na+ channels toward cocaine and bupivacaine optical isomers. Tonic inhibitions of Na+ currents by 200 μM (-)cocaine, 300 μM (+)cocaine, 100 μM (-)bupivacaine, and 100 μM (+)bupivacaine after 3-min treatment are shown in A, B, C, and D, respectively. The control currents immediately before drug treatment were superimposed in the figure. Notice that under the concentrations used, ~40–60% of peak Na+ currents were inhibited. The holding potential was set at −100 mV, and the test pulse was +30 mV for 5 ms. After drug treatment, repetitive depolarizations further reduce the Na+ currents to <20% of the control level as shown in the bottom traces of A–D. Each pair of isomers at the concentrations indicated blocked about the same amount of Na+ current. The current scales for each cell are shown. The test pulses were +30 mV for 30 ms and were applied repetitively for a total of 30 pulses at 2 Hz. Notice that the peak current reached its steady-state level within 5–10 pulses.

**Dose–Response Relationship of Cocaine and Bupivacaine Stereoisomers**

The tonic inhibitory effect of cocaine and bupivacaine stereoisomers on Na+ currents is concentration dependent (Fig. 3 A). The tonic action of (-)cocaine is relatively rapid; it reaches its steady-state inhibition within 2–3 min. Fig. 3 B shows the rapid time course of inhibition by (-)cocaine at various concentrations. The rapid action of (-)cocaine therefore permits us to measure the dose–response relationship accurately. The wash-off of the drug is equally rapid; nearly complete recovery can be achieved within 2–3 min. The dose–response curve at $E_H = -100$ mV is shown in Fig. 4 A. The data can be fitted with the Langmuir isotherm (dotted line, open
circles) with a $K_D$ value of 194 µM for (-)-cocaine. Similar rapid time courses of inhibition and recovery by (+)-cocaine and by (+) and (-)bupivacaine were found (data not shown). The dose–response curves of these drugs are also plotted in Fig. 4, A and B (dotted lines). Table I lists the $K_D$ values of these drugs at $E_H = -100$ mV. The difference of stereoselectivity of (+)/(-)-cocaine and (+)/(-)-bupivacaine is less than a factor of 2 in the tonic block of normal Na$^+$ currents.

![Figure 3](image)

**Figure 3.** The time course of the inhibitory action of (-)-cocaine on Na$^+$ currents. (A) The current traces recorded at various (-)-cocaine concentrations were superimposed. The numeric values indicate the (-)-cocaine concentration applied in micromolar. The test pulse was +30 mV for 5 ms, and holding potential was set at -100 mV. The dashed line is the zero current baseline. (B) The normalized peak Na$^+$ currents as in A were normalized and plotted against time. The conditions of superfusion of external (-)-cocaine at various concentrations are shown in the upper portion of the graph (boxed). Notice that the peak current nearly reaches its steady-state level within 2 min of drug treatment. Washing with drug-free solution quickly removes the cocaine effect, also within 2 min. The solid line in B was drawn by eye.

The Potency of Cocaine and Bupivacaine Isomers Can Be Modulated by a Prolonged Prepulse

Holding potential or long prepulse potential can affect the potency of LAs (Courtney, 1975; Hille, 1977). Typical neuronal cells in vivo have a resting potential of approximately -70 mV. With this prepulse potential for 5 s, both cocaine and bupivacaine stereoisomers were found to be more potent than their counterparts at the holding potential of -100 mV by about four- to fivefold (Fig. 4, A and B; solid lines). The $K_D$ value at $E_{pp} = -70$ mV of each isomer is listed in Table I. The dose–response curves again can be well fitted by the Langmuir isotherm (Fig. 4, solid
Stereoselectivity of Local Anesthetics in Na⁺ Channels

\[ \frac{I_{Na}}{I_{Na}^0} = \frac{K_D}{K_D + [L]} \]

(line). We conclude that the binding affinity of these stereoisomers in GH3 cells can be modulated by the long prepulse potential. It is interesting to note here that the stereoselectivity of both cocaine and bupivacaine remains minimal at \( E_{pp} = -70 \) mV.

**Shifts of \( h_R \) by Cocaine Stereoisomers**

It has been reported that the extent of resting Na⁺ channel inactivation is increased after treatment of various LAs (e.g., Hille, 1977). Fig. 5 shows that both (+) and (−) cocaine enhance the steady-state Na⁺ channel inactivation (\( h_R \)) as revealed by a
two-pulse protocol. Pulses were applied at a rate of every 30 s in drug-treated cells because the recovery time constant for the block was relatively slow, ~2–3 s. Shifts of ~10 and 20 mV toward the hyperpolarizing direction were found for 400 μM (+)cocaine and 300 μM (-)cocaine, respectively. At these concentrations, ~60–70% of the Na⁺ current was inhibited. Under such conditions, the binding reactions were likely to reach a steady state within the 50-ms duration of a given prepulse. The steepness of the \( h_{\text{m}} \) curve was reduced by (+) and (-) cocaine (the slope factor in Fig. 5 was altered from 8.43 to ~11 mV/e-fold change). These results can be interpreted to mean that the inactivated Na⁺ channels are "stabilized" by the LA molecule and hence fewer resting Na⁺ channels are available for opening (for details, see Hille, 1977).

### Table 1

| Optical isomers | \( K_0 \) [\( E_m = -100 \text{ mV} \)] | \( K_0 \) [\( E_m = -70 \text{ mV} \)] | (+)/(-) ratio |
|-----------------|-------------------|-------------------|-----------------|
|     \(-\)Cocaine | \( 195 \pm 12 \mu M \) | \( 60.8 \pm 3.4 \mu M \) |                |
|     \(+\)Cocaine | \( 361 \pm 30 \mu M \) | \( 95.5 \pm 4.9 \mu M \) | 1.85            |
| \(-\)Bupivacaine | \( 107 \pm 10 \mu M \) | \( 25.9 \pm 2.4 \mu M \) | 1.50            |
| \(+\)Bupivacaine | \( 139 \pm 9 \mu M \) | \( 16.4 \pm 1.5 \mu M \) | 0.63            |

The values of the equilibrium dissociation constant \( (K_0) \) of each optical isomer were obtained by the best-fitted curve according to the Langmuir isotherm shown in Fig. 4. Na⁺ currents were assayed at +30 mV.

**Voltage Dependence of the Use-dependent Block by (+) and (-)Cocaine**

Since the \( h_{\text{m}} \) curves were shifted toward a hyperpolarizing direction by LA isomers, we further investigated whether repetitive depolarizations at conditioning potentials without Na⁺ channel opening could elicit any significant use-dependent block. After a 500-ms recovery interval (Fig. 6A), a test pulse was applied to determine the amount of block produced by the repetitive depolarizations. The 500-ms recovery interval was long enough to allow unblocked channels to recover from fast and slow inactivation, but short enough to allow only ~15% recovery from cocaine-induced block. We found that a 60% reduction of the peak Na⁺ current at the test pulse was obtained after repetitive pulses of -50 mV for 30 ms at 2 Hz (Fig. 6). It is noteworthy that at -50 mV few or no Na⁺ currents are activated (Fig. 1), whereas >80% of normal Na⁺ channels will be inactivated at this conditioning pulse (Fig. 5). In contrast, Crumb and Clarkson (1990) showed that an extensive overlap occurred in the voltage dependence of the use-dependent block by (-)cocaine and in the activation of cardiac Na⁺ channels. The reason for this different result is not known. Our result suggests that in GH3 cells cocaine stereoisomers need not bind to the
open channel to elicit the use-dependent block. In other words, cocaine isomers can directly interact with inactivated channels. Such an interpretation would be consistent with the results shown in Fig. 4, where we demonstrated that the binding affinity is higher for cocaine isomers at −70 mV than at −100 mV holding potential. Taken together, our results indicate that cocaine and bupivacaine isomers can directly interact with resting, open, and inactivated states of normal Na⁺ channels. However, during depolarization these isomers may interact mostly with the inactivated state,
which is the predominant state. The following diagram summarizes these interactions:

![Diagram](image)

Figure 6. Voltage dependence of the use-dependent block elicited by (+)/(−) cocaine. (A) Pulse protocol and representative current traces. Use-dependent block was elicited by a train of 30 conditioning pulses ($E_c$) of 30 ms duration at 2 Hz. The remaining peak Na$^+$ current after the conditioning train was measured with a test pulse ($E_{test} = +30$ mV for 10 ms) after a recovery interval of 500 ms. Current traces represent before (left) and after 400 μM (+)cocaine treatment with $E_c$ from −100 to −40 mV with a 10-mV increment. Arrows indicate current traces with $E_c = −40$ mV. (B) Open circles show the control peak current amplitude ($n = 8$ cells) after normalization with the peak current value at $V_c = −180$ mV. Closed circles are data measured under the same conditions with 300 μM (−)cocaine present ($n = 4$ cells); closed triangles show data with 400 μM (+)cocaine present ($n = 4$ cells). Note that the control peak current decreases when $E_c \geq −20$ mV. This is due to the combined slow inactivation of Na$^+$ currents occurring sequentially during repetitive pulses. When complete recovery of slow inactivation was allowed to take place between different $E_c$ pulses, little inhibition of Na$^+$ currents occurred after a repetitive pulse.
Evidently, the stereoselectivity of LAs in the resting (C) and inactivated (I) states of Na⁺ channels toward these isomers (L) is minimal. The stereoselectivity of LAs in the open state (O) will be addressed below.

**Effects of Cocaine and Bupivacaine Isomers on BTX-modified Na⁺ Currents**

BTX is known to keep the Na⁺ channel open during a prolonged depolarization. Fig. 7A shows the BTX-modified Na⁺ current at +50 mV (control). The current is maintained during a 5-s pulse at +50 mV. Upon application of (-)cocaine at 3–300 μM, the BTX-modified Na⁺ current is inhibited in a time-dependent manner. Again, the blocking effect of cocaine reaches its steady state within 2–3 min. The time-dependent block is presumably due to a higher binding affinity of (-)cocaine at +50 mV than at the holding potential (with a -130-mV prepulse for 5 s). It is likely that (-)cocaine (even at 300 μM) does not interact significantly, if at all, with the resting channels as judged by the fact that little block occurs at the beginning of the +50 mV pulse, whereas a >90% block by 300 μM (-)cocaine is found at the end of the pulse (Fig. 7A). In contrast, application of (+)cocaine at 10 μM to 1 mM gives rise to a much lesser inhibition of Na⁺ current (Fig. 7B). To make sure that the lesser effect of (+)cocaine is not due to the presence of leak currents we applied 2 μM TTX. Evidently, there is little leak current contributed in the current trace (Fig. 7B). Our results thus demonstrate directly that the stereoselectivity ratio of (+)/(-) cocaine is altered drastically in BTX-modified Na⁺ channels.

Like that of (+)/(-)cocaine, the stereoselectivity ratio of (+)/(-)bupivacaine in BTX-modified Na⁺ channels is very different from that in normal Na⁺ channels. Fig.
8A shows that (−)bupivacaine at 3–30 μM induces a relatively strong time-dependent block as (−)cocaine (Fig. 7A). In contrast, at 30 μM (+)bupivacaine the inhibition of Na⁺ current is slight, usually <15% (Fig. 8B). However, at 300 μM (+)bupivacaine the Na⁺ current is inhibited more and a time-dependent block becomes evident. Again, (+)/(−) bupivacaine stereoselectivity is enhanced to reach ~30-fold. Clearly, the stereoselectivity of both cocaine and bupivacaine in BTX-modified Na⁺ channels is very different from that in normal Na⁺ channels, thus supporting the idea that the altered stereoselectivity in the presence of BTX is a common phenomenon. These results also show that (−)cocaine interacts preferentially with the open BTX-modified Na⁺ channels but poorly, if at all, with the closed form as shown in the following diagram:

\[
\begin{align*}
C \cdot \text{BTX} & \leftrightarrow O \cdot \text{BTX} \\
& \downarrow \text{(−)cocaine} \\
O \cdot \text{BTX} \cdot \text{(−)cocaine}
\end{align*}
\]

where C is the closed form and O is the open form of BTX-modified Na⁺ channels.

Dose–Response Relationship of Cocaine and Bupivacaine Stereoisomers in BTX-modified Na⁺ Currents

For approximation, the inhibitory effect of stereoisomers was measured at the end of a 5-s pulse at +50 mV. At this time point, the inhibitory effect of stereoisomers nearly reached its maximum. Fig. 9 shows the dose–response curve of (−) and (+)cocaine by these measurements. The data can be well described by the Langmuir isotherm and suggest that one cocaine molecule blocks one BTX-modified Na⁺ channel as previously demonstrated in bilayer studies (Wang, 1988). The estimated \( K_0 \) values are listed in Table II. The stereoselectivity ratio of these LAs is relatively high, compared with that in normal Na⁺ channels (Table I).

Effects of Cocaine and Bupivacaine Isomers on CT-modified Na⁺ Currents

CT is an oxidant that is known to modify Na⁺ channel inactivation (Wang, 1984). To test whether the open state of Na⁺ channels has a different stereoselectivity for cocaine and bupivacaine isomers, we treated the GH3 cell with 0.5 mM external CT for 10 min. In the presence of CT, inactivation of Na⁺ channels is progressively removed and most of the Na⁺ current is maintained during a 4-ms pulse. Prolonged depolarization of the membrane, however, slowly inactivates the CT-modified Na⁺ channel with a time constant of ~1.5 s at +50 mV (Fig. 10). This result indicates that the intermediate and/or ultra-slow inactivation of Na⁺ channels in GH3 cells is not removed by the CT treatment as previously demonstrated in myelinated nerve fiber (Ulbricht and Stoye-Herzog, 1984) and in squid axons (Wang, Brodwick, Eaton, and Strichartz, 1987). Both (−) and (+)bupivacaine inhibit Na⁺ currents in CT-treated cells but the stereoselectivity is not significantly altered as compared with that in unmodified Na⁺ currents (cf. Figs. 2 and 10). Quantitatively, the two stereoisomers
are about equally potent in CT-treated GH3 cells (Fig. 10, A and B). In addition, a relatively fast time-dependent block of the CT-modified Na⁺ current is also evident for (−) and (+)bupivacaine, indicating that the open state has a higher binding affinity than the resting state. This experiment was repeated in three separate GH3 cells with comparable findings. Similarly, (+)/(−) cocaine stereoselectivity in CT-treated Na⁺ channels is not altered compared with that of normal Na⁺ channels (data

![Figure 8](image)

**Figure 8.** Stereoselectivity of BTX-modified Na⁺ channels toward bupivacaine optical isomers. (A) BTX-modified Na⁺ current traces at +50 mV before (Control) and after superfusion with concentrations of (−)bupivacaine (micromolar) at 3, 10, and 30 were superimposed and labeled numerically. (B) A separate cell was treated with (+)bupivacaine at concentrations of 30, 100, and 300 µM. Notice that (−) and (+)bupivacaine are more potent than (−) and (+)cocaine (Fig. 7), respectively. A pre-pulse of −130 mV for 5 s was applied before each test pulse.

![Figure 9](image)

**Figure 9.** The dose–response curve of cocaine isomers in BTX-modified Na⁺ channels. The inhibitory effect of cocaine was measured at the end of a 5-s pulse at +50 mV (see Fig. 7), normalized with the control current amplitude at the same time point, and plotted against the concentration. The inhibitory effects of (−)cocaine (open circles) and (+)cocaine (closed circles) can be well fitted with the Langmuir isotherm (solid lines), suggesting that one cocaine molecule blocks one BTX-modified Na⁺ channel. Error bars represent measurements from three separate cells at each data point.
Stereoselectivity of Cocaine and Bupivacaine in the Time-dependent Inhibition of BTX-modified Na⁺ Currents

| Optical isomers | $K_D$ at +50 mV (μM) | (+)/(−) ratio |
|-----------------|----------------------|---------------|
| (−)Cocaine      | 35.8 ± 2.6           |               |
| (+)Cocaine      | 661.2 ± 65.0         | 18.5          |
| (−)Bupivacaine  | 7.0 ± 1.5            |               |
| (+)Bupivacaine  | 222.4 ± 24.8         | 31.8          |

The $K_D$ values of each optical isomer were determined as shown in Fig. 7. All data were best-fitted by the Langmuir isotherm.

not shown). It is likely that CT, unlike BTX, does not alter the conformation of the LA receptor so that the LA binding as well as the LA stereoselectivity is not changed.

**DISCUSSION**

This report examines the stereoselectivity of LA drugs toward neuronal Na⁺ channels in intact GH₃ cells with and without BTX present. We have found that the stereoselectivity of cocaine and bupivacaine optical isomers toward BTX-modified Na⁺ channels is indeed different from that toward unmodified counterparts. In addition, we have explored the underlying mechanism of this altered stereoselectivity phenomenon. Both cocaine and bupivacaine interact directly with the open state of BTX-modified Na⁺ channels. However, interactions of these LA drugs with the closed state of BTX-modified Na⁺ channels are hampered. Detailed discussions on these and other related subjects follow.

**FiguRe 10.** The inhibitory effect of Na⁺ currents by bupivacaine isomers in CT-treated GH₃ cells. The normal fast inactivating Na⁺ currents were converted to CT-modified Na⁺ currents after 10 min of 0.5 mM external CT treatment. The cell was then washed with CT-free solution. (A) Superimposed current traces are shown before (Control) and after (−)bupivacaine treatment at 3, 10, and 30 μM (labeled). Notice that a time-dependent block of Na⁺ currents by (−)bupivacaine is evident. (B) The same cell was washed with drug-free solution before (Control) and after (+)bupivacaine treatment at 3, 10, and 30 μM (labeled). (−)Bupivacaine is about as potent as (+)bupivacaine. A prepulse of −130 mV for 0.5 s was applied before each test pulse.
Validity of Bilayer Studies on the Stereoselectivity of LA Drugs toward BTX-modified Na⁺ Channels

The concerns about the validity of the bilayer studies on the high LA stereoselectivity toward Na⁺ channels could not be easily addressed in our previous report (Wang, 1990). First, could the synthetic lipids used in planar bilayers affect the LA stereoselectivity? Second, could the stereoselectivity be modulated by diffusible cytosolic factors in intact cells? And finally, what is the stereoselectivity ratio in intact cells? To answer these questions, we have extended bilayer studies to neuronal intact GH3 cells. The results show that in rat GH3 cells cocaine stereoselectivity (~20-fold) toward neuronal BTX-modified Na⁺ channels remains the same as in brain BTX-modified Na⁺ channels (also 20-fold; Wang, 1990). Thus, artificial bilayers alone do not give rise to the relatively high stereoselectivity; intact cells show the similar high stereoselectivity. These experiments also indicate that the lipid environment (phosphatidylethanolamine [PE]/phosphatidylcholine [PC] = 2/1) of the incorporated Na⁺ channels is not critical for LA binding or for LA stereoselectivity. In contrast, Tamkun, Talvenheimo, and Catterall (1984) reported that α-scorpion toxin does not bind to purified rat brain Na⁺ channels reconstituted in egg phosphatidylcholine vesicles but binds well when channels are reconstituted in rat brain lipids, suggesting that the lipid environment is important for α-scorpion toxin and Na⁺ channel interactions. The fact that LA binding is insensitive to the Na⁺ channel lipid environment is perhaps due to the location of the LA receptor, which is proposed to be within the Na⁺ permeation pathway (Wang, Simon, and Wang, 1991). Furthermore, there is no evidence for diffusible cytosolic factors that may modulate the stereoselectivity ratio in intact cells, despite the fact that modulations of Na⁺ channels by cAMP-dependent protein kinase A and by cAMP receptors have been reported (Costa, Casnellie, and Catterall, 1982; Numann, Catterall, and Scheuer, 1991; Sorbera and Morad, 1991).

Different LA Stereoselectivity in Normal and BTX-modified Na⁺ Channels

It has not been reported before that in intact cells the LA stereoselectivity in BTX-modified Na⁺ channels is different from that in normal Na⁺ channels. We found that the GH3 cell preparation is suitable for this particular study. The cell has a round shape and is relatively small (~20–40 μm in diameter). Most of normal Na⁺ currents in GH3 cells can be easily converted to the BTX-modified current after ≤5–10 min of repetitive stimulations. Using these neuronal cells, we have now demonstrated that in normal Na⁺ channels the LA stereoselectivity is indeed weak, as reported in other preparations (for review, see Courtney and Strichartz, 1987), whereas in BTX-modified Na⁺ channels the LA stereoselectivity is relatively high as in muscle, brain, and cardiac BTX-modified Na⁺ channels incorporated into planar bilayers (Wang, 1990). One noteworthy abnormality is that (−)-bupivacaine is more potent than (−)-cocaine in normal and BTX-modified Na⁺ channels from rat neuronal GH3 cells, whereas the reverse is true in BTX-modified Na⁺ channels from rabbit skeletal muscle (this report vs. Wang, 1990). This result suggests that the potency of a given LA drug may be tissue specific. Similarly, we have also noticed that in bilayers (−)-cocaine is four times more potent in calf cardiac BTX-modified Na⁺
channels than in rabbit muscle BTX-modified Na⁺ channels (Wang, 1988). It would be of interest to examine whether this differential tissue block is present within one single species.

What Is the Underlying Mechanism for the Altered Stereoselectivity in BTX-modified Na⁺ Channels?

Previously we suggested two possible explanations for the altered stereoselectivity by BTX. First, we speculated that there may be more than one LA receptor in Na⁺ channels as reported before (e.g., Mrose and Ritchie, 1978; Huang and Ehrenstein, 1981; for review, see Courtney and Strichartz, 1987). Second, we speculated that the open channel conformation may have different stereoselectivity than the closed channel conformation. Neither possibility can be substantiated by this study. The dose-response curves of the tonic block of both cocaine and bupivacaine in normal Na⁺ channels are fitted well by the Langmuir isotherm (Figs. 3 and 4), suggesting a one-to-one relationship in the ligand-receptor interactions. Furthermore, normal unmodified Na⁺ channels and channels that remain open during prolonged depolarization after the pretreatment of CT exhibit similar weak stereoselectivity toward LAs. Clearly, an increase in the open probability of Na⁺ channels by CT does not lead to the altered stereoselectivity. What then is the underlying mechanism for this phenomenon? It seems appropriate now to hypothesize that a conformational change induced by BTX occurs within the region of the LA binding site. This allosteric mechanism between LAs and BTX binding sites has been proposed previously by Postma and Catterall (1984). As a result of this conformational change at the LA binding site, stereoselectivity of the LA in Na⁺ channels could also be altered. If this hypothesis is correct, LA stereoselectivity in Na⁺ channels may be also affected by other activators of Na⁺ channels such as veratridine and aconitine. We are now testing this possibility in GH3 cells under patch clamp conditions.

State-dependent Binding of LA Drugs with Normal and BTX-modified Na⁺ Channels

Although it was demonstrated in bilayer experiments that both cocaine and bupivacaine block muscle BTX-modified Na⁺ channels in their open conformation, the question of whether these LA drugs can also interact with the resting form of the BTX-modified Na⁺ channels was not addressed. The results shown in Figs. 7 and 8 suggest that cocaine and bupivacaine do not interact strongly, if at all, with the resting BTX-modified Na⁺ channels (see Scheme B). However, when the activation gate of BTX-modified Na⁺ channels is open, the drug interacts strongly with the open channels and hence elicits the apparent time-dependent block during a prolonged depolarization. Although at relatively high cocaine concentration an inhibition of the peak BTX-modified Na⁺ current becomes evident (Fig. 7), this inhibition could be due to a very fast time course of LA block on open Na⁺ channels.

In contrast, clear tonic block of normal Na⁺ currents by cocaine and bupivacaine is observed in GH3 cells (Figs. 3 and 4). Obviously, LAs can block the resting states of normal Na⁺ channels more easily than their BTX-modified counterparts (Scheme A vs. B). More than 70% of normal Na⁺ currents can be tonically blocked by cocaine and bupivacaine isomers at 1 mM, even if the cell is held at −100 mV. Nonetheless, these drugs may have a higher affinity for the open and inactivated states of normal channels than the resting counterparts for the following reasons. First, the use-
dependent block is present for these drugs after repetitive depolarizations. At small repetitive depolarizations (from \(-90\) to \(-50\) mV) the additional block is perhaps due to the drug binding with the inactivated Na\(^+\) channels (Fig. 6). At large repetitive depolarizations where the channels can be activated, the use-dependent block is perhaps also due to additional binding of these drugs with the open state of Na\(^+\) channels (Fig. 6; see also Strichartz, 1973; Wang et al., 1987). Second, the time-dependent block by these drugs is found to occur in the CT-modified Na\(^+\) currents (Fig. 10). This result is consistent with the notion that cocaine and bupivacaine interact more strongly with the open state than with the resting state of Na\(^+\) channels.

The differences in the tonic inhibition of normal and BTX-modified Na\(^+\) currents by these drugs may provide clues for why so many reports suggest that the binding of LA drugs is abolished or diminished by the activators (reviewed by Khodorov, 1978). Most of the previous results were assayed by a relatively short depolarization (i.e., <50 ms). The time-dependent block may be less conspicuous for such a short pulse (Figs. 7 and 8). Clearly, precautions should be taken in studying the LA effects on BTX-modified Na\(^+\) currents. Steady-state conditions cannot be reached by depolarization with a short duration; prolonged depolarization is needed to observe the LA-induced block in BTX-modified Na\(^+\) currents.

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