Binding of Benzocaine in Batrachotoxin-modified Na+ Channels

State-dependent Interactions

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ABSTRACT   Hille (1977. *Journal of General Physiology*. 69:497–515) first proposed a modulated receptor hypothesis (MRH) to explain the action of benzocaine in voltage-gated Na^+ channels. Using the MRH as a framework, we examined benzocaine binding in batrachotoxin (BTX)-modified Na^+ channels under voltage-clamp conditions using either step or ramp command signals. We found that benzocaine binding is strongly voltage dependent. At -70 mV, the concentration of benzocaine that inhibits 50% of BTX-modified Na^+ currents in GH_3 cells (IC_{50}) is 0.2 mM, whereas at $+50$ mV, the IC_{50} is 1.3 mM. Dose-response curves indicate that only one molecule of benzocaine is required to bind with one BTX-modified Na^+ channel at -70 mV, whereas approximately two molecules are needed at $+50$ mV. Upon treatment with the inactivation modifier chloramine-T, the binding affinity of benzocaine is reduced significantly at -70 mV, probably as a result of the removal of the inactivated state of BTX-modified Na^+ channels. The same treatment, however, enhances the binding affinity of cocaine near this voltage. External Na^+ ions appear to have little effect on benzocaine binding, although they do affect cocaine binding. We conclude that two mechanisms underlie the action of local anesthetics in BTX-modified Na^+ channels. Unlike open-channel blockers such as cocaine and bupivacaine, neutral benzocaine binds preferentially with BTX-modified Na^+ channels in a closed state. Furthermore, benzocaine can be modified chemically so that it behaves like an open-channel blocker. This compound also elicits a use-dependent block in unmodified Na^+ channels after repetitive depolarizations, whereas benzocaine does not. The implications of these findings for the MRH theory will be discussed.

INTRODUCTION

Benzocaine is a neutral local anesthetic (LA) that contains no tertiary amine component. In contrast, other clinical LAs, such as bupivacaine, lidocaine, and cocaine, possess a tertiary amine that can be protonated at physiological pH (Ritchie

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and Greene, 1985). The action of benzocaine and tertiary amine LAs on Na^+ currents and gating currents has been extensively studied (for review, see Keynes, 1983; Butterworth and Strichartz, 1990; Hille, 1992). It has been suggested that benzocaine and tertiary amine LAs share a common receptor and bind more strongly with inactivated Na^+ channels than with their resting and open counterparts (Hille, 1977; 1992). This hypothesis is generally referred to as Hille's modulated receptor hypothesis (MRH).

Attempts to validate Hille's hypothesis have had mixed results. On the one hand, removal of Na^+ channel inactivation by pronase treatment appears to reduce the potency of the quaternary derivative of lidocaine, QX-314, on Na^+ currents, as would be predicted by the MRH theory (Cahalan, 1978; Yeh, 1978). On the other hand, the conflicting results obtained from the modification of Na^+ channel inactivation by α-scorpion toxin or by chloramine-T (CT) suggest that inactivation is not a prerequisite for LA action (Shepley, Strichartz, and Wang, 1983; Wang, Brodwick, Eaton, and Strichartz, 1987; Ulbricht and Stoye-Herzog, 1984). Thus, whether Na^+ channel inactivation is directly involved in LA action remains an open question.

Recently, two distinct modes of action of LAs in batrachotoxin (BTX)-modified Na^+ channels have been reported. First, tertiary amine LAs, such as cocaine and bupivacaine, have been found to be open-channel blockers; these LAs do not interact with closed BTX-modified Na^+ channels (Wang, 1988; Wang and Wang, 1992b). Second, preliminary results indicate that benzocaine binds to closed BTX-modified Na^+ channels and interacts less effectively with the open channel (Moczydlowski Uehara, Guo, and Heiny, 1986; Wang and Wang, 1992a). A similar observation was reported by Ulbricht and Stoye-Herzog (1984), who found that benzocaine cannot effectively block veratridine-modified Na^+ channels in the open state but binds strongly to channels in their closed state. An unfortunate complication in studies of the action of benzocaine is that the dose-response curve for benzocaine binding in unmodified Na^+ channels cannot be fitted by the Langmuir isotherm. At an intermediate dose range (0.25–1 mM), the binding of one benzocaine molecule appears to block one Na^+ channel, whereas at a high dose range (1–2 mM) two benzocaine molecules are probably needed (Meeder and Ulbricht, 1987; Elliott and Haydon, 1989).

The purpose of this study was to examine benzocaine binding with BTX-modified Na^+ channels in the closed and open states using the whole-cell patch clamp technique. The planar lipid bilayer technique is not suitable for this purpose because, for reasons unknown to us, single BTX-modified Na^+ channels often disappear in the presence of benzocaine (Moczydlowski et al., 1986). Treatment with BTX allows us to distinguish the inactivated state of BTX-modified Na^+ channels in GH_3 cells from the resting and open states (Wang and Wang, 1992a). Without BTX, it is extremely difficult to study inactivated Na^+ channels because they rarely reopen during depolarization. The results of this study led us to suggest that neutral benzocaine preferentially binds to BTX-modified Na^+ channels in a closed form (probably the inactivated state). Furthermore, we also found that it is possible to convert the closed-channel blocker benzocaine into an open-channel blocker by simple structural modification. The ramifications of these findings for specific aspects of Hille's MRH theory will be discussed.

MATERIALS AND METHODS

Chemicals

Benzocaine (pKa = 2.54) was purchased from Sigma Chemical Co. (St. Louis, MO). N-dimethyl benzocaine (ethyl p-dimethylamino-benzoate) was obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). Both compounds were dissolved in dimethylsulfoxide at 500 mM and maintained as stock solutions at 4°C. CT was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was dissolved in H_2O at 40 mM before use. BTX and (−)-cocaine were provided by Dr. John Daly and Dr. Rao Rapaka (National Institutes of Health, Bethesda, MD), respectively.

Benzocaine   **N-dimethyl Benzocaine**

(−) Cocaine
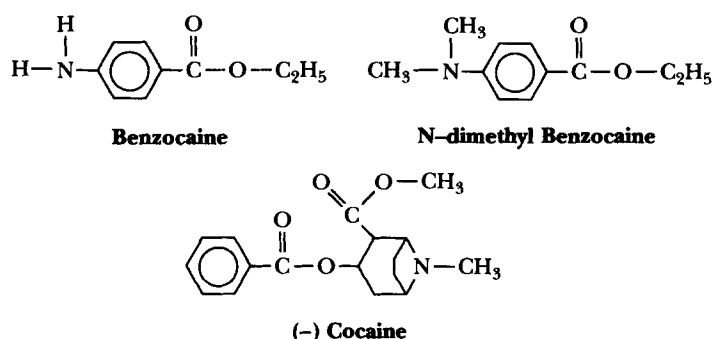
Cell-Culture and Whole-Cell Voltage Clamp

Rat pituitary clonal GH_3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1X glutamine as described by Cota and Armstrong (1989). The whole-cell variant of the patch-clamp method (Hamill, Neher, Sakmann, and Sigworth, 1981) was used to measure Na^+ currents in GH_3 cells. Two external solutions were used; 0 Na^+ solution, which contains (in mM) 150 choline·Cl, 0.2 $CdCl_2$, 2 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with tetramethyl hydroxide (TMA-OH), and 130 Na^+ solution, which contains 20 choline·Cl, 130 NaCl, 0.2 $CdCl_2$, 2 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with TMA-OH.

Micropipettes were fabricated and had a tip resistance of ~1 MΩ when filled with a high Na^+ solution containing (in mM): 0.005 BTX, 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. The junction potential of electrodes was nulled before seal formation. Liquid junction potentials were measured to be 3.5 ± 0.2 mV and 1.6 ± 0.2 mV (n = 4) for 130 Na^+ and 0 Na^+ external solution, respectively, and were not corrected in this report. After the rupture of the patch membrane, repetitive pulses (+50 mV with 50 ms at 2 Hz; Khodorov, 1978) for 5–10 min, converted normal channels into BTX-modified Na^+ channels (60–95%), as judged by the ratio of maintained (at 50 ms) and peak (at ~1 ms) Na^+ currents. Data acquisition and analysis were undertaken as described before (Wang and Wang, 1992a). Voltage-ramp protocols were created with pClamp software (Axon Instruments, Inc., Foster City, CA). BTX-modified Na^+ currents generated by this pulse protocol were easily measurable. Leak and capacitance were subtracted by a homemade device. All experiments were performed at room temperature (23 ± 2°C). At the end of experiments, the drift in the junction potential was generally < 2 mV. Results of analyses are presented as mean ± standard error.

RESULTS

Only One Benzocaine Binding Site in the Inactivated BTX-modified Na+ Channel

Previously, we suggested that 1 mM benzocaine strongly promotes the inactivation of BTX-modified Na^+ channels at a membrane potential near the resting potential (i.e., -70 mV) (Wang and Wang, 1992a). To determine the number of benzocaine binding site(s) responsible for the enhancement of inactivation of BTX-modified Na^+ channels, we examined the dose-response relationship. Fig. 1 *A* shows BTX-modified Na^+ currents before and after treatment with various concentrations of benzocaine. After a 5-s prepulse to -70 mV, the control BTX-modified Na^+ current exhibits a biphasic rising phase during a test pulse of $+50$ mV, as has previously been reported. The initial rising phase (too fast to be recorded at this time scale) was interpreted as representing the opening of resting BTX-modified Na^+ channels and the second (slow) rising phase as representing the reopening of inactivated BTX-modified Na^+ channels populated during the -70 mV prepulse (Epp). Such a reopening process
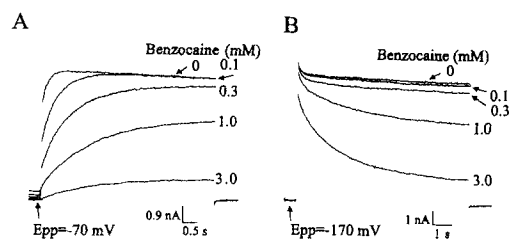

FIGURE 1. Inhibition of BTX-modified Na^+ currents by benzocaine. Na^+ channels were activated by a test pulse of $+50$ mV with a prepulse (*Epp*) of -70 mV (*A*) or -170 mV (*B*) for 5 s. Current traces were superimposed for comparison. Benzocaine (at the concentration indicated) was applied for 1–2 min while the cell was held at -100 mV. With an Epp of -70 mV, the biphasic rising phase of Na^+ currents is present (*A*), whereas only a rapid rising phase, too fast to be recorded, is present with a prepulse of -170 mV (*B*). Na^+ currents recorded during an Epp of -70 mV were also inhibited by benzocaine in a concentration-dependent manner (*A;* front end of current traces).

from normal inactivated Na^+ channels at $+50$ mV occurs rarely, if at all. Treatment with benzocaine not only slows the kinetics of the second rising phase but also progressively reduces the amplitude of the first rising phase in a concentration-dependent manner. On the assumption that the reduction of Na^+ currents in the first rising phase is due to direct binding of benzocaine during the Epp of -70 mV (Fig. 1 *A*) and that the remaining current is from benzocaine-free BTX-modified Na^+ channels, we constructed a dose-response curve. Fig. 2 (*open circle*) shows the data obtained from seven separate cells, which can be well fitted by a Langmuir isotherm (*solid line*, consistent with a Hill coefficient value of 0.99 ± 0.02). The concentration that inhibits 50% of the initial phase of the Na^+ current (IC_{50}) was estimated to be 207 ± 12 µM. This result suggests that at -70 mV only one benzocaine molecule is needed to block one BTX-modified Na^+ channel. The action of benzocaine is readily reversible; superfusion of GH_3 cells with benzocaine-free solution reverses the effects of benzocaine on BTX-modified Na^+ channels within 1 min. It is therefore unlikely that the inhibition of currents shown in Fig. 1 *A* is due to the dissociation of BTX from its binding site while benzocaine is present. If BTX dissociated from its receptor, the BTX-modified Na^+ current would not reappear after washing unless

repetitive pulses were reapplied for the reassociation of BTX with the open state of normal Na^+ channels (Tanguy and Yeh, 1991).

Deviation from the One-to-One Relationship for Benzocaine Block in BTX-modified Na^+ Channels at +50 mV

Contrary to inactivated BTX-modified Na^+ channels, their resting and open counterparts do not appear to bind to benzocaine effectively (Fig. 1 *B*). When resting Na^+ channels (at -170 mV) are activated by a strong depolarizing pulse (+50 mV), benzocaine elicits a time-dependent block that is concentration dependent. The degree of block at the end of the pulse was measured and plotted against the benzocaine concentration. Surprisingly, the dose-response relationship is not consistent with a simple bimolecular interaction. The channel block by benzocaine is much too steep to be fitted by a Langmuir isotherm (Fig. 2, *dashed line*); instead a Hill coefficient of 1.6 ± 0.1 is observed (Fig. 2, *solid circles*). The IC_{50} is estimated to be
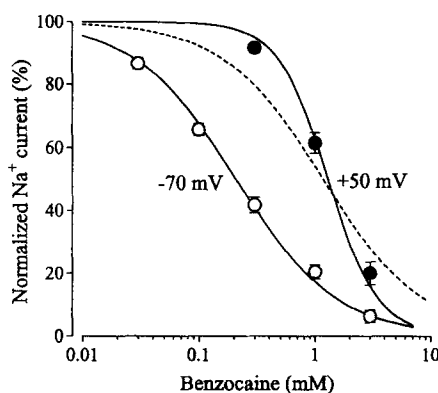
FIGURE 2.   Dose-response curve for benzocaine inhibition of BTX-modified Na^+ currents. The amplitudes of the fast rising phase of Na^+ currents (shown in Fig. 1 *A*) at various benzocaine concentrations were measured, normalized in relation to the control-current amplitude, and plotted against the benzocaine concentration (*open circle*) ($n = 7$). The solid line represents the theoretical curve with a Hill coefficient of 1. The steady state currents maintained at the end of the test pulse were similarly analyzed in Fig. 1 *B* (*solid circle*) ($n = 5$). Bar symbols represent standard errors. In three other experiments, steady state currents as shown in Fig. 1 *B* were also analyzed and yielded similar results. The solid line represents the theoretical curve with a Hill coefficient of 2, the dashed line with a coefficient of 1.

1.3 ± 0.1 mM. The solid line shown in Fig. 2 at +50 mV is the theoretical curve with a Hill coefficient of 2.0.

It is also possible to measure the channel block at +50 mV from the re-equilibration of the benzocaine-bound inactivated Na^+ channels. The unblock, as indicated by a slowed rising phase (shown in Fig. 1 *A*) suggests that benzocaine binds to the open BTX-modified Na^+ channels with a lesser affinity than to the inactivated channels. The current amplitude at the end of the pulse was measured, normalized in relation to the control current amplitude, and plotted against the benzocaine concentration. It should be noted here that drug binding to, and unbinding from channels at +50 mV nearly, but not completely, reach steady state during a 5- to 10-s pulse as shown in Fig. 1. Unfortunately, GH_3 cells rarely survive 15- to 30-s depolarizing pulses to +50 mV. The dose-response curve obtained by this method yields an IC_{50} value of 1.1 ± 0.1 mM and a Hill coefficient of 1.6 ± 0.1, nearly

identical to those shown in Fig. 2. Binding of benzocaine with resting BTX-modified channels at -170 mV is extremely weak, as indicated by the small reduction of Na^+ currents at the beginning of the depolarization in Fig. 1 B ($IC_{50} > 3$ mM). Because of the poor solubility of benzocaine at concentrations higher than 3 mM, no attempts were made to fit the dose-response curve for the benzocaine binding with resting BTX-modified Na^+ channels.

Voltage-dependent Inhibition of BTX-modified Na^+ Currents by Benzocaine

In further studies of the voltage dependence of benzocaine binding, we applied a voltage-ramp protocol ranging from -130 mV to $+50$ mV. Fig. $3A$ shows BTX-
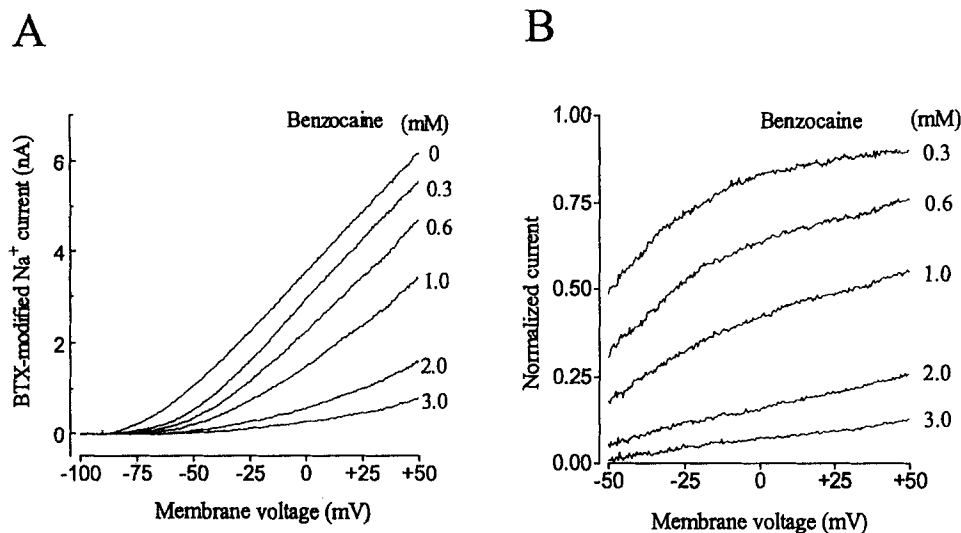
A   B

FIGURE 3. Voltage dependence of benzocaine binding in BTX-modified Na^+ channels. BTX-modified Na^+ currents (A) were activated by a voltage-ramp protocol from -130 mV to $+50$ mV linearly for 15 s. The current trace was truncated from -130 to -100 mV where no Na^+ currents were present. Benzocaine (at the concentrations indicated) was applied for 1–2 min while the cell was held at -100 mV. The degree of block was greater at the resting membrane potential (-50 to -90 mV) than at positive potentials (0 to $+50$ mV). The amplitudes of Na^+ currents in (A) at various benzocaine concentrations at each voltage were measured, normalized in relation to the control-current amplitude, and plotted against the voltage (B). Because of the relatively small current amplitudes below -50 mV, no reliable normalization can be obtained. Results were similar in five other cells.

modified currents under this voltage-ramp protocol in the absence and presence of benzocaine at various concentrations. Without benzocaine, the BTX-modified current is normally activated between -90 mV and -80 mV, and the current amplitude then increases continuously in a nearly linear manner. The shape of the current-voltage ramp relationship is essentially the same as that of the current-voltage relationship measured previously by step depolarizations (Wang and Wang, 1992a). In the presence of benzocaine, the inhibition of BTX-modified Na^+ currents is clearly voltage dependent (but see Schneider and Dubois, 1986). At the voltage range

around −90 to −60 mV of the current voltage-ramp curve, most currents are blocked by this range of benzocaine concentrations (0.3–3 mM), whereas at the more positive voltage potentials, the currents are inhibited less.

This pulse protocol allows us to examine the apparent voltage-dependent binding visually from −50 to +50 mV. It is interesting that IC_{50} continues to increase from 0.3 mM at −50 mV to ~1.2 mM at +50 mV (Fig. 3 B). There is no sudden switch from a high binding affinity to a lower affinity within this voltage range. The dose-response curve at +50 mV is indistinguishable to that measured by voltage jump experiments shown in Fig. 2. The current-voltage ramp relationship remains the same when the duration of voltage ramp is 15 s (Fig. 3 A) or longer (i.e., 1–2 min). This observation suggests that drug binding reaches its quasi steady-state (but not equilibrium) under this condition.

Lack of Reduction of Benzocaine Binding by External Na+ Ions

It is commonly known that external Na+ ions can reduce the binding affinity of tertiary and quaternary amine LAs. To assess whether external Na+ ions affect neutral benzocaine binding, we raised the external Na+ ion concentration from 0 mM to 130 mM. Fig. 4 A shows that the inward Na+ current is first activated around −90 to −80 mV, reaches its peak, and then reverses (to outward) near its reversal potential, 5.8 ± 0.8 mV (n = 14), which is slightly higher than the theoretical value of 0 mV. The reason for this ~6-mV depolarizing shift in the reversal potential is not clear but may be due in part to the uncorrected liquid junctional potential between internal and external solutions and the incomplete dialysis of cytoplasmic fluid with the pipette solution. Inward Na+ currents were activated near −90 mV threshold voltage, which is ~50 mV more negative than the activation threshold of normal Na+ currents (Fig. 5). In the presence of benzocaine, there is an apparent voltage-dependent shift of the peak current-voltage ramp relationship toward a more positive voltage direction (Fig. 4 and 5). The inward Na+ current is now activated at larger depolarizations, reaches its peak at more positive potentials, and reverses near its reversal potential. The degree of block by benzocaine is again strongly dependent on the voltage applied (Fig. 4 B). The dose-response curve can be constructed with data from this type of experiment within the voltage range from −75 mV up to +50 mV. Fig. 6 shows that at −70 mV the data can be well fitted by a one-to-one relationship (with a Hill coefficient of 0.97 ± 0.02), whereas at +50 mV they are again fitted by approximately a two-to-one relationship (with a Hill coefficient of 1.6 ± 0.1). The estimated IC_{50} values at the 130 mM external Na+ ion concentration (0.14 ± 0.01 mM and 1.12 ± 0.01 mM at −70 mV and +50 mV, respectively) are slightly lower than those without external Na+ ions (Fig. 2 and Fig. 3 B). Hence, external Na+ ions do not reduce benzocaine binding. This result is in sharp contrast with the binding of tertiary and quaternary LAs with Na+ channels reported before in squid axons, in lipid bilayers, and in cardiac myocytes (Cahalan and Almers, 1979; Wang, 1988; Barber, Wendt, Starmer, and Grant, 1992) as well as in GH3 cells (Wang, G.K., unpublished observation).

State-dependent Binding of Benzocaine in BTX-modified Na+ Channels

It has been proposed that benzocaine binds preferentially to the inactivated Na+ channels (Hille, 1977; 1992). How does one demonstrate that this hypothesis is
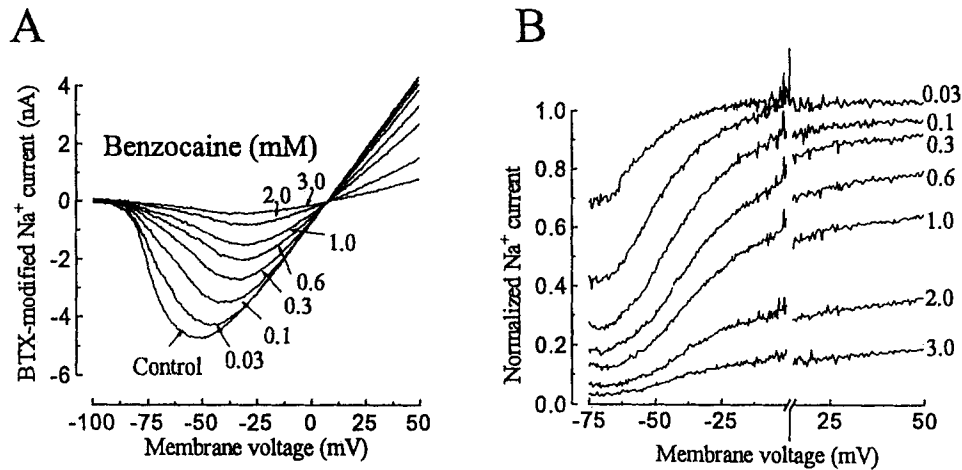
FIGURE 4. Effects of external Na^+ ions on benzocaine binding in BTX-modified Na^+ channels. Na^+ currents were activated by a pulse protocol identical to that in Fig. 3 A. The external solution contained 130 mM of Na^+ ions, the same concentration as the internal solution in the patch pipette. The inward Na^+ current was activated at around −85 mV (A). Benzocaine at various concentrations was applied for 1–2 min while the cell was held at −100 mV. After benzocaine treatment the I/V curve was shifted toward a more positive direction and currents were inhibited in a voltage-dependent manner. Na^+ current amplitudes at various benzocaine concentrations were measured at voltages from −75 to +50 mV, normalized in relation to the control-current amplitude, and plotted against voltage (B). Normalized currents near the reversal potential (+6.8 mV) were truncated because of difficulties in normalization due to their small amplitudes.
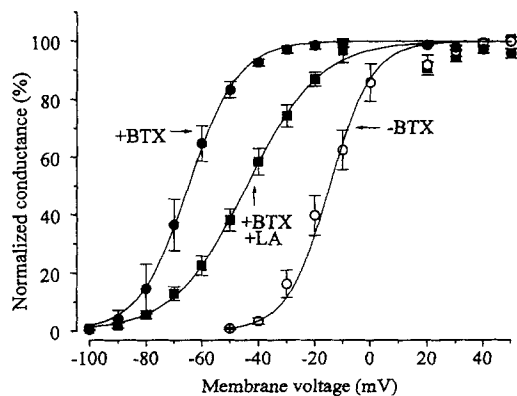

FIGURE 5. Voltage dependence of normalized Na^+ conductance before (○) and after (●) BTX treatment. Conductance (g) was determined from the equation $g = I_{Na}$ $(V\text{-}V_{rev})$ where I_{Na} was the current amplitude under voltage-ramp conditions (as shown in Fig. 4 A) or the peak I_{Na} when no BTX was present. The conductance was normalized with respect to the maximal conductance and plotted against voltage. The solid lines were a least-squares fit of the data and were drawn according to an equation:

$y = 100/\{1 + \exp[(V_{0.5} - V)/k]\}$ where the voltage for half-maximal activation, $V_{0.5}$, is −64 ± 0.5 mV ($n = 5$) and −14.6 ± 1.4 mV ($n = 4$), and the steepness factor, k, is 8.9 ± 0.4 mV ($n = 5$) and 7.6 ± 0.5 mV ($n = 4$) for BTX-modified and normal Na^+ channels, respectively. Further treatment of BTX-modified Na^+ channels with 1 mM benzocaine (■; i.e., +BTX + LA) shifted the $V_{0.5}$ by about 20 mV toward the depolarizing direction ($V_{0.5} = -44.0 ± 0.9$ mV, $n = 4$) and altered the steepness factor, $k = 12.8 ± 0.8$ mV ($n = 4$).
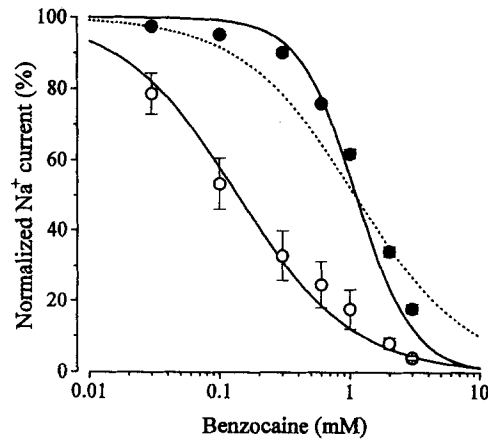
FIGURE 6. Voltage dependence of the dose-response curve at a 130 mM external Na$^+$ ion concentration. The normalized current amplitudes at −70 and +50 mV, as shown in Fig. 4 B were plotted against the benzocaine concentration. Data were obtained from four separate cells. The Hill coefficient for the dose-response curve was estimated to be 0.97 at −70 mV (the solid line represents the theoretical curve with a Hill coefficient of 1) and 1.6 at +50 mV (the solid line represents the theoretical curve with a Hill coefficient of 2, whereas the dashed line with a Hill coefficient of 1). Notice that the potency of benzocaine at high external Na$^+$ solution is similar to that without external Na$^+$ (Fig. 2).

indeed the mechanism for the action of benzocaine in BTX-modified Na$^+$ channels? To address this question, we further applied CT to remove the inactivation of BTX-modified Na$^+$ channels (Wang and Wang, 1992a). Fig. 7 shows current traces under voltage ramp protocol before and after CT treatment for 5 min. After CT treatment, BTX-modified Na$^+$ channels are activated at more negative potentials (a left-shift of 15 ± 1.4 mV, $n = 7$), consistent with the findings reported previously in mammalian preparations (Cukierman, 1991; Niemann, Schmidtmayer, and Ulbricht, 1991). Current amplitudes also increase slightly between −50 and +50 mV. The degree of the current increase varies from cell to cell perhaps because CT-modified Na$^+$ channels (with an increase in the open probability) bind to BTX more efficiently than unmodified channels (Tanguy and Yeh, 1991). This modification of the
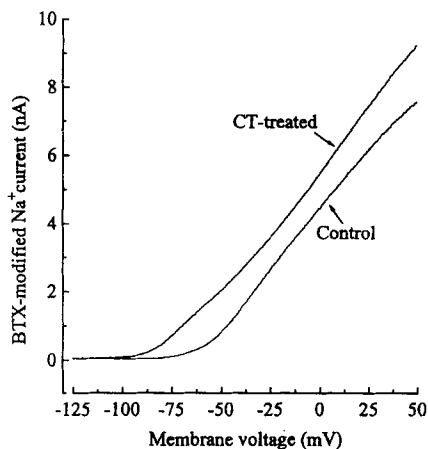

FIGURE 7. Modification of current-voltage ramp relationship by CT. Na$^+$ currents were generated by a standard voltage ramp protocol identical to that described in Fig. 3 A. BTX-modified Na$^+$ currents were measured before and after treatment with 0.5 mM CT for 5 min.

current-voltage ramp curve by CT is irreversible, even after continued superfusion of cells with drug-free solution.

After application of CT the level of benzocaine binding is greatly reduced as measured from −75 mV to +50 mV (Fig. 8, C and D vs Fig. 8, A and B). Changes in the shape of the current-voltage ramp curve are also observed; benzocaine no longer binds strongly at the more negative potentials (i.e., at −50 mV; Fig. 8, B vs D). In fact, the reduction of binding at −50 mV is significantly greater than that at +50 mV
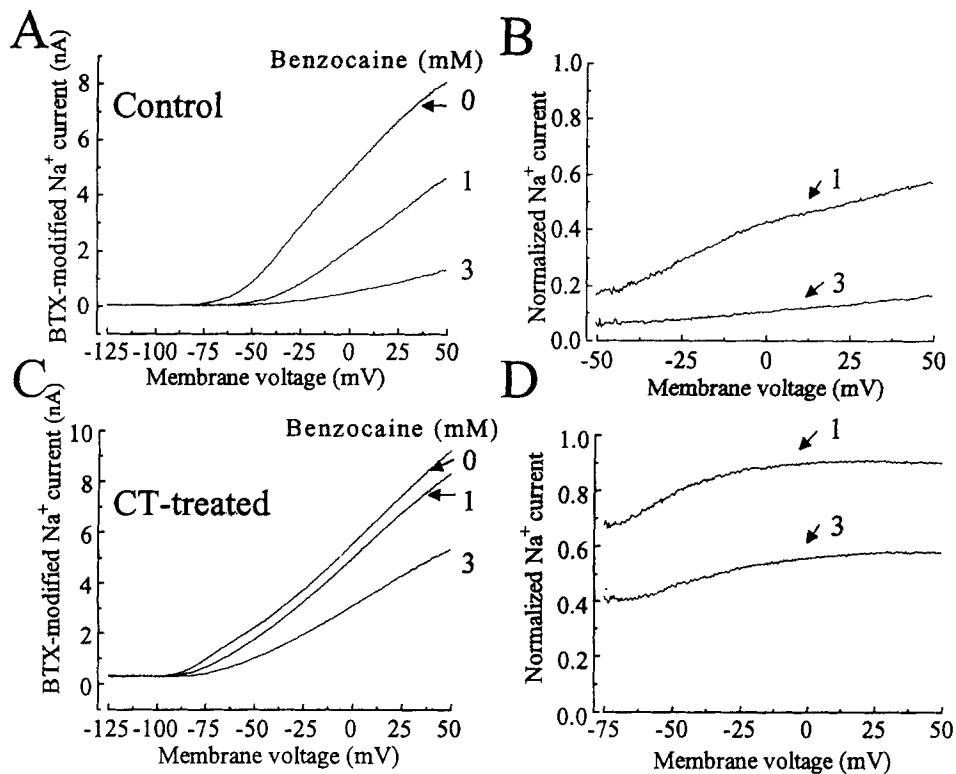

FIGURE 8.   Reduction in benzocaine binding after treatment with CT. The effect of benzocaine (1 and 3 mM) on the current-voltage ramp relationship was measured before (A) and after (C) CT-treatment (0.5 mM, 5 min) with a standard voltage ramp protocol. Current amplitudes in the presence of benzocaine were normalized in relation to the control-current amplitude and plotted against voltage (B and D). CT treatment reduces the binding affinity of benzocaine and changes the voltage dependence of this binding. Results were similar in seven other cells.

(Fig. 8, B vs D). We were unable to construct dose-response curves of benzocaine in the CT-treated cells due to the reduced benzocaine affinity and the poor solubility of benzocaine. Nevertheless, our result provides strong evidence that, upon binding, benzocaine enhances the inactivation of BTX-modified Na^+ channels.

It is puzzling to observe the result shown in Fig. 8, because CT treatment does not affect benzocaine binding with unmodified Na^+ channels in myelinated nerve fibers (Ulbricht and Stoye-Herzog, 1984; Meeder and Ulbricht, 1987). A closer examina-

tion of Fig. 8 *C* at the voltage range between −95 mV and −60 mV shows that benzocaine still blocks BTX-modified Na⁺ currents substantially more than at voltages between −50 and +50 mV (Fig. 8 *D*). The higher benzocaine affinity at voltages between −95 mV and −60 mV could be due in part to incomplete removal of inactivation by CT and in part to a higher affinity of benzocaine toward the resting BTX-modified Na⁺ channels. Because a prolonged treatment with CT usually leads to membrane instability, we did not attempt to perform such an experiment.
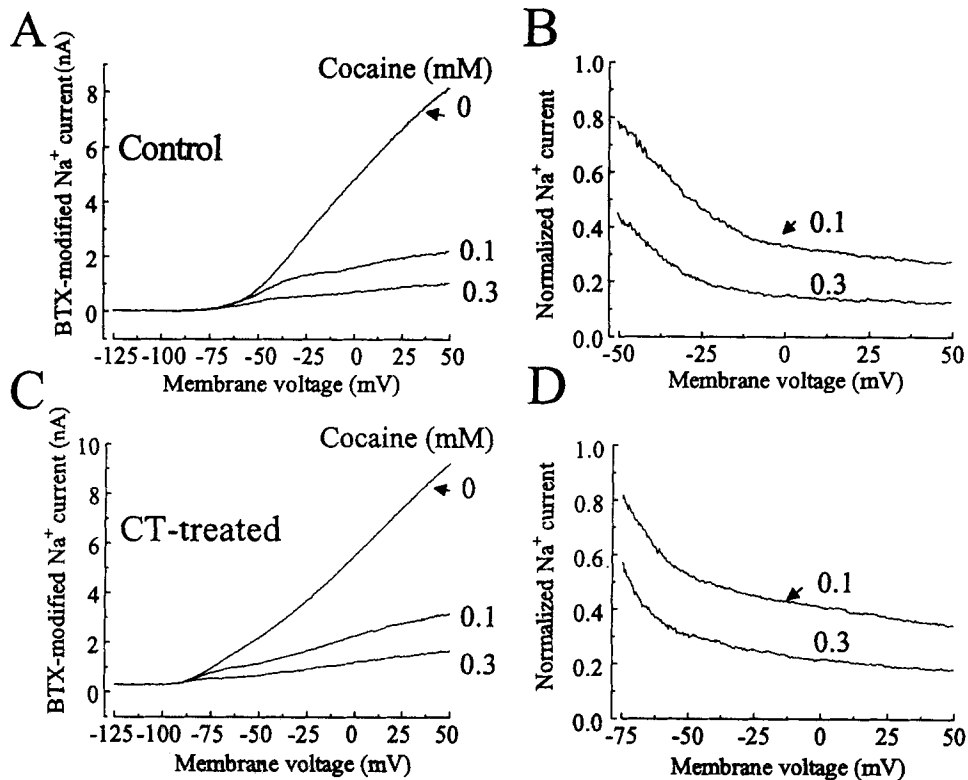

FIGURE 9. Effects of CT treatment on cocaine binding. Cocaine at 0.1 mM or 0.3 mM was applied before (*A*) and after (*C*) treatment with 0.5 mM CT for 5 min. A small reduction in cocaine binding was found after CT treatment (*B*) and (*D*) at voltages from 0 mV to +50 mV, whereas an increase in block was found from −50 mV to −25 mV. Results were similar in three other cells.

Binding of Cocaine in BTX-modified Na⁺ Channels After CT Treatment

Because it can be argued that treatment with CT nonspecifically affects LA receptors in the Na⁺ channel structure, we repeated the protocol in the presence of cocaine. We found that treatment with CT affects the binding of cocaine in a manner opposite to that seen with benzocaine (Fig. 9). After CT treatment cocaine binding is slightly enhanced from −50 mV to −30 mV (Fig. 9, *B* vs *D*), probably because this LA has a

greater probability of interacting with the open channels due to the left shift in the *I/V* relationship. Evidently, cocaine does not interact well with inactivated BTX-modified Na^+ channels, because its binding is relatively weak at -50 mV, particularly in cells without CT treatment (Fig. 9 *B*). In addition, the voltage-dependent binding of cocaine shows a pattern that is the opposite of that seen with benzocaine. Inhibition of Na^+ currents by cocaine under quasi steady state voltage-ramp conditions is significantly stronger at $+50$ mV than at -50 mV. There is a small reduction in binding from 0 to $+50$ mV after CT treatment (Fig. 9 *D*), but this reduction is minimal compared with the reduction in benzocaine binding at -50 mV (Fig. 8 *D*). Hence, treatment with CT results in a specific reduction of benzocaine binding but in little or no reduction of cocaine binding.

Conversion of Benzocaine to a Different Mode of Action

The different modes of action of benzocaine and cocaine in BTX-modified Na^+ channels raise the possibility that an inactivation enhancer can be converted to a blocker with a different state-dependent binding characteristic by modification of its
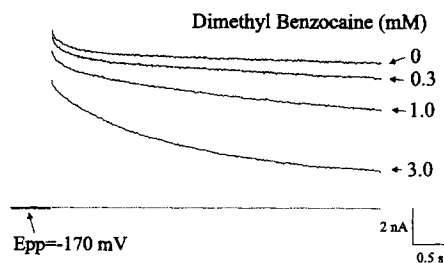

FIGURE 10. Effects of *N*-dimethyl benzocaine on BTX-modified Na^+ currents. *N*-dimethyl benzocaine at various concentrations was applied for 1–2 min while the cell was held at -100 mV. The external solution contained no Na^+ ions. Step depolarization to $+50$ mV (with a prepulse of -170 mV) activated BTX-modified Na^+ currents. *N*-dimethyl benzocaine elicited a time-dependent block that was concentration dependent. This experiment was repeated in four other cells, which yielded similar results.

structure. We chose several benzocaine derivatives to test this possibility. Among them were ethyl 4-hydroxy benzoate, ethyl 3-hydroxy benzoate, tricaine, methyl 4-amino benzoate, and *N*-dimethyl benzocaine. One of the derivatives, *N*-dimethyl benzocaine, failed to enhance the inactivation of BTX-modified Na^+ channels, whereas the remaining drugs acted more or less as benzocaine. Fig. 10 shows that *N*-dimethyl benzocaine, like benzocaine, produces a clear time-dependent block during a $+50$-mV test pulse, a result suggesting that the resting channel binds poorly with this drug. We were unable to study the dose-response curve in detail, because we could not reliably dissolve *N*-dimethyl benzocaine at 3 mM even after heating. However, *N*-dimethyl benzocaine appears to block BTX-modified Na^+ channels in a different voltage-dependent manner (Fig. 11 *A*) from that of benzocaine under voltage-ramp conditions. The degree of block at various voltages was measured and plotted against voltage. Block of *N*-dimethyl benzocaine shows a voltage dependence that is reversed from (i.e., enhanced by depolarization) and weaker than the voltage dependence of benzocaine block. Hence, *N*-dimethyl benzocaine has no clear ability

to promote the inactivated state of BTX-modified Na⁺ channels nor to block efficiently the resting state (Fig. 10). It is interesting that external Na⁺ ions also have no effect on the binding of N-dimethyl benzocaine; the blocking effects of this benzocaine derivative shown in Fig. 11 B are comparable to those seen at a 130-mM external Na⁺ concentration (data not shown). These results together suggest that it is possible to convert benzocaine to a different state-dependent blocker by simple structural modification and that external Na⁺ ions may not affect the binding of this converted blocker when the active form of the drug is not charged.
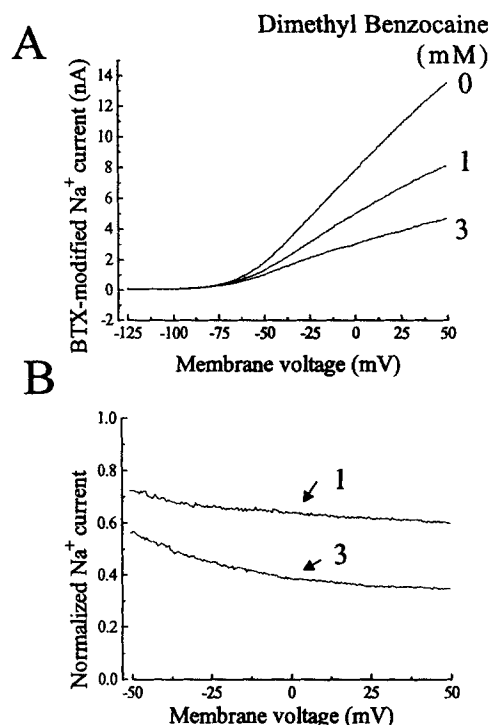

FIGURE 11. Voltage dependence of N-dimethyl benzocaine binding. Na⁺ currents were activated by a standard voltage-ramp pulse protocol. N-dimethyl benzocaine at the concentration indicated was applied for 1–2 min while the cell was held at −100 mV (A). Current amplitudes with 1 and 3 mM dimethyl benzocaine at various voltages were measured, normalized with relation to the control level, and plotted against voltage (B). The voltage-dependent binding of N-dimethyl benzocaine is different from that of benzocaine (Fig. 8 B). The external solution contained no NaCl.

Use-dependent Block of N-Dimethyl Benzocaine in Unmodified Na⁺ Channels

It is unclear that the differences between benzocaine and N-dimethyl benzocaine in blocking BTX-modified Na⁺ channels have any application to unmodified Na⁺ channels. Indeed, BTX modifies most of Na⁺ channel properties including channel gating, ion selectivity, as well as other ligand binding properties (Khodorov, 1978). Therefore, we chose to examine the use-dependent block of benzocaine and N-dimethyl benzocaine in unmodified Na⁺ channels as a possible indicator for the existence of different blocking mechanisms.

Fig. 12 A shows that benzocaine at 1 mM produces a tonic block of normal Na⁺ currents but fails to elicit additional use-dependent block when stimulated at a frequency of 5 Hz (Fig. 12 C). In contrast, N-dimethyl benzocaine at 1 mM not only

produces a tonic block (Fig. 12 B) but also elicits additional use-dependent block of Na$^+$ currents (Fig. 12 D). Furthermore, without a -130-mV prepulse (Fig. 12 C), Na$^+$ current amplitude is much smaller than that with a prepulse (Fig. 12 A). This effect is due to a left shift in steady-state inactivation (h_∞) of ~ 20 mV by 1 mM benzocaine (also see Hille, 1977). The left shift is much smaller by 1 mM dimethyl benzocaine (~ 10 mV). Clearly, N-dimethyl benzocaine and benzocaine behave quite differently in blocking normal as well as BTX-modified Na$^+$ channels.
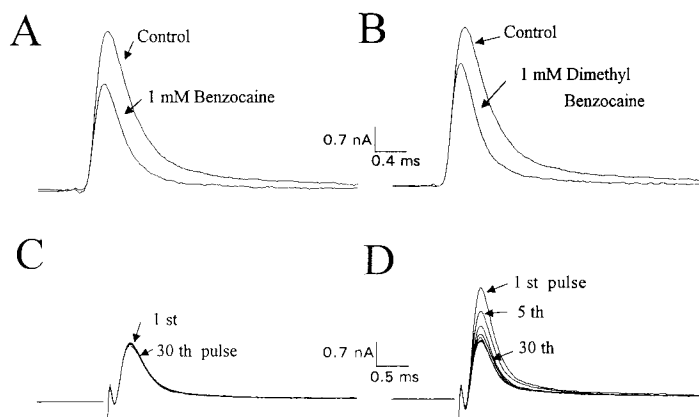

FIGURE 12.   Use-dependent block of normal Na$^+$ channels by N-dimethyl benzocaine. Normal Na$^+$ channels in the absence of BTX were activated by a $+30$ mV depolarizing test pulse before and after treatment of 1 mM benzocaine (A) or 1 mM N-dimethyl benzocaine (B). The holding potential was -100 mV, and the prepulse potential of -130 mV for 50 ms was applied before the test pulse. Use-dependent block was further developed after repetitive test pulses ($+30$ mV for 25 ms at a frequency of 5 Hz) for N-dimethyl benzocaine (D) but not for benzocaine (C). Current traces were recorded every fifth pulse and superimposed for comparison. No prepulses were applied during repetitive test pulses. Leak and capacitance in (C) and (D) were subtracted partially by analog device. The external solution contained no NaCl.

DISCUSSION

This study describes four new findings: (a) Only one benzocaine molecule is needed to enhance the inactivation of one BTX-modified Na$^+$ channel. (b) External Na$^+$ ions have little or no effect on benzocaine binding. (c) Removal of the inactivation of BTX-modified Na$^+$ channels by CT drastically reduces their binding affinity for benzocaine near -70 mV. (d) A benzocaine derivative, N-dimethyl benzocaine, fails to enhance the inactivation of BTX-modified Na$^+$ channels. Block of BTX-modified Na$^+$ channels by N-dimethyl benzocaine exhibits a different voltage dependence from that of benzocaine. Also, N-dimethyl benzocaine, but not benzocaine, elicits a use-dependent block in unmodified Na$^+$ channels. These findings, along with the deviation from the one-to-one relationship of benzocaine binding with BTX-modified Na$^+$ channels at $+50$ mV, will now be discussed in detail.

Interaction of Benzocaine with Inactivated BTX-modified Na^+ Channels

Because the dose-response curve for benzocaine at −70 mV can be well fitted by a Langmuir isotherm with IC_{50} that is lower than other voltages and because inactivation is maximal around this potential (Wang and Wang, 1992a), we surmise that only one benzocaine molecule is needed to enhance the inactivation of one BTX-modified Na^+ channel. If so, then inactivation modifiers theoretically would reduce benzocaine binding. This conjecture is strongly supported by the fact that treatment with CT reduces the binding affinity of benzocaine for BTX-modified Na^+ channels. The effect of CT treatment on benzocaine binding at −70 mV appears to be specific. We do not find a drastic reduction of cocaine binding at −70 mV when we test the same CT-treated cell; in fact, cocaine binding is actually enhanced around this voltage (Fig. 9). Removal of inactivation, therefore, has a much less pronounced effect on cocaine binding.

Previously, Ulbricht and his coworkers (Ulbricht and Stoye-Herzog, 1984; Meeder and Ulbricht, 1987) reported that benzocaine binding to unmodified Na^+ channels remains practically unchanged after 1 min of CT treatment. Their results are clearly different from our finding in BTX-modified Na^+ channels shown in Fig. 8, *B* and *D*. However, we also noted that benzocaine still binds, although with a reduced affinity, around −95 mV to −75 mV more strongly than around −50 to +50 mV in BTX-modified Na^+ channels (Fig. 8 *C*). It is possible that unmodified Na^+ channels in their resting state and inactivated state have a similar affinity toward benzocaine. If so, CT treatment may produce no significant changes in the binding affinity of benzocaine in unmodified Na^+ channels.

Two-to-One Stoichiometry of Benzocaine Binding in BTX-modified Na^+ Channels at +50 mV

At +50 mV, BTX-modified Na^+ channels display not only a weaker binding affinity for benzocaine but also a nonunity stoichiometry in binding with benzocaine (Figs. 4 and 6). The simplest explanation for our dose-response curve is that at +50 mV two benzocaine molecules block one BTX-modified Na^+ channel. If this is true, an additional benzocaine binding site must become available when the membrane voltage is switched to +50 mV. Both benzocaine binding sites must be occupied if Na^+ permeability is to be inhibited.

Alternatively, it is possible that a nonlinear relationship exists between the aqueous concentration of benzocaine and the effective concentration near the binding site. (For a discussion of this possibility, see Meeder and Ulbricht, 1987.) To explain the dose-response curve for BTX-modified Na^+ channels at +50 mV, one would need to postulate that the effective benzocaine concentration increases more than the aqueous benzocaine concentration in a nonlinear manner. Finally, benzocaine may cause membrane thickening (Elliott, Haydon, and Hendry, 1984; Elliott and Haydon, 1989), which in turn may cause an abnormality in benzocaine binding with BTX-modified channels. Unfortunately, these two alternatives do not explain why only one benzocaine molecule is needed to block BTX-modified Na^+ channels at −70 mV when tested at the same concentration range.

Structural Determinant of Benzocaine in the Enhancement of Inactivation

How benzocaine enhances the inactivation of BTX-modified Na^+ channels and why N-dimethyl benzocaine fails to do so are important questions that need to be addressed. Because N-dimethyl benzocaine does not enhance the inactivation of BTX-modified Na^+ channels but does block open Na^+ channels, a simple explanation for this behavior is that the 4-amino group of benzocaine is involved in the enhancement of inactivation upon binding. This 4-amino functional group is either dimethylated as in N-dimethyl benzocaine or not present as in (−)cocaine. The interactions between the 4-amino group and its binding site could stabilize the inactivated state of BTX-modified Na^+ channels because they do not reopen readily (Fig. 1). One type of interaction may be through hydrogen bond(s) formed between the 4-amino group of benzocaine and its receptor. Alternatively, the unshared electron of the 4-amino group may alter the resonance characteristic of the phenyl moiety, which upon binding may stabilize the receptor. These various possibilities may be addressed by detailed studies of the structure-activity relationship of benzocaine and its homologs.

It is interesting that we observed no effects of external Na^+ ions on neutral benzocaine or N-dimethyl benzocaine binding. Many studies have suggested that the LA binding site is within the Na^+ permeation pathway, because external Na^+ ions can reduce the binding affinity of tertiary and quaternary LAs (Cahalan and Almers, 1979; Wang, 1988; Barber et al., 1991). One obvious explanation for the lack of effect of Na^+ ions is that both benzocaine and N-dimethyl benzocaine are neutral compounds. Hence, no charge repulsion between Na^+ ions and neutral LAs will develop even if they encounter each other within the permeation pathway.

Implications for Hille's MRH as a Working Model

Hille's MRH for LA binding in unmodified Na^+ channels consists of two general principles (Hille, 1977). First, there is one common binding site for all LAs. Second, this binding site changes its configuration in a state-dependent manner. Hille further proposed that Na^+ channels in the inactivated state bind most strongly with LAs. As a result, such a conformation is stabilized upon binding. The following discussion is based on the MRH model as adapted for BTX-modified Na^+ channels.

The idea of a common binding site in Na^+ channels for all LAs remains controversial (for a review see Butterworth and Strichartz, 1990). If two benzocaine molecules are indeed needed to block one BTX-modified Na^+ channel at +50 mV, then, by definition, two benzocaine binding sites must exist. Except in one case (Fig. 2), however, all LAs in our studies blocked BTX-modified Na^+ channels in a one-to-one relationship (e.g., Wang and Wang, 1992b). At present, if we are to retain the one common binding site for all LAs as a working model, we are left with one last possibility: that the common LA binding site in BTX-modified Na^+ channels may accommodate two benzocaine molecules simultaneously at +50 mV. This possibility may be feasible, because the LA binding site has been found to contain two large hydrophobic binding domains (Wang, 1990). Without a protonatable amine, the benzocaine molecule is unlikely to repel the other even when they are adjacent to each other. Our results, however, do not permit us to distinguish this possibility from

the two-site theory. Other approaches, such as competition experiments in single BTX-modified Na$^+$ channels or photoaffinity labeling and site-direct mutagenesis of cloned Na$^+$ channels may yield unequivocal evidence for one of these hypotheses.

To address the second component of Hille's MRH, we have demonstrated that benzocaine binding is indeed strongly state dependent. This state-dependent binding can be modified by the inactivation modifier CT. The specific feature of Hille's MRH that LAs bind most strongly with the inactivated form of Na$^+$ channels, therefore, is consistent with benzocaine binding in BTX-modified Na$^+$ channels. However, this is not the case for open-channel blockers of BTX-modified Na$^+$ channels in planar lipid bilayers or in GH$_3$ cells (Moczydlowski et al., 1986; Wang and Wang, 1992b). Evidently, two mechanisms underlie the action of LAs in BTX-modified Na$^+$ channels. As a result, LAs may bind preferentially with the closed form of BTX-modified Na$^+$ channels (benzocaine) or with the open form (cocaine). In the future, different modes of LA action may also be uncovered in unmodified Na$^+$ channels. The clear use-dependent block elicited only by N-dimethyl benzocaine in unmodified Na$^+$ channels but not by benzocaine may serve as a useful indicator to support such a conclusion.

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