Charged Tetracaine as an Inactivation Enhancer in Batrachotoxin-Modified Na^+ Channels

G. K. Wang,* W. M. Mok,* and S-Y Wang‡
*Department of Anesthesia Research Laboratories, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115; and ‡Department of Biology, State University of New York at Albany, Albany, New York 12222 USA

ABSTRACT Two distinct types of local anesthetics (LAs) have previously been found to block batrachotoxin (BTX)-modified Na^+ channels: type 1 LAs such as cocaine and bupivacaine interact preferentially with open channels, whereas type 2 LAs, such as benzocaine and tricaine, with inactivated channels. Herein, we describe our studies of a third type of LA, represented by tetracaine as a dual blocker that binds strongly with closed channels but also binds to a lesser extent with open channels when the membrane is depolarized. Enhanced inactivation of BTX-modified Na^+ channels by tetracaine was determined by steady-state inactivation measurement and by the dose-response curve. The 50% inhibitory concentration (IC_{50}) was estimated to be 5.2 μM at −70 mV, where steady-state inactivation was maximal, with a Hill coefficient of 0.98 suggesting that one tetracaine molecule binds with one inactivated channel. Tetracaine also interacted efficiently with Na^+ channels when the membrane was depolarized; the IC_{50} was estimated to be 39.5 μM at +50 mV with a Hill coefficient of 0.94. Unexpectedly, charged tetracaine was found to be the primary active form in the blocking of inactivated channels. In addition, external Na^+ ions appeared to antagonize the tetracaine block of inactivated channels. Consistent with these results, *N*-butyl tetracaine quaternary ammonium, a permanently charged tetracaine derivative, remained a strong inactivation enhancer. Another derivative of tetracaine, 2-(dimethylamino) ethyl benzoate, which lacked a 4-butylamino functional group on the phenyl ring, elicited block that was ~100-fold weaker than that of tetracaine. We surmise that 1) the binding site for inactivation enhancers is within the Na^+ permeation pathway, 2) external Na^+ ions antagonize the block of inactivation enhancers by electrostatic repulsion, 3) the 4-butylamino functional group on the phenyl ring is critical for block and for the enhancement of inactivation, and 4) there are probably overlapping binding sites for both inactivation enhancers and open-channel blockers within the Na^+ pore.

INTRODUCTION

Batrachotoxin (BTX) is an alkaloid neurotoxin that drastically alters the gating properties of voltage-dependent Na^+ channels in excitable membranes. Activation is shifted by ~30–60 mV in a hyperpolarizing direction, whereas inactivation is largely eliminated (for reviews see Khodorov, 1978; Catterall, 1980; Strichartz et al., 1987; Hille, 1992a). However, several reports indicate that steady-state inactivation still occurs in BTX-modified Na^+ channels in neuronal cells (Zubov et al., 1984; Mozhayeva et al., 1986) and in cardiac myocytes (Huang et al., 1987; Wasserstrom et al., 1993). In a recent study, we found that the neutral local anesthetics (LAs) benzocaine and tricaine profoundly enhance the inactivation of BTX-modified Na^+ channels in rat pituitary clonal GH_3 cells, particularly near the potential range between −90 and −40 mV (Wang and Wang, 1992a; 1994; but see Schneider and Dubois, 1986, in frog nerve fibers). Beyond this range, the block of benzocaine is weakened; this observation is consistent with the notion that benzocaine does not interact strongly with the resting and the open states of BTX-modified Na^+ channels.

In contrast to benzocaine and tricaine, a group of LAs including QX-314 (a derivative of lidocaine), cocaine, bupivacaine, and their derivatives (Moczydlowski et al., 1986; Wang, 1988) as well as hydrophilic and amphipathic quaternary ammonium (QA) compounds (Moczydlowski et al., 1986; Green et al., 1987; Wang et al., 1991) appear as open-channel blockers. These drugs interact minimally with the closed state of BTX-modified Na^+ channels in GH_3 cells. Thus, there are two types of blockers for BTX-modified Na^+ channels in GH_3 cells: type 1 interacts preferentially with the open state, and type 2 interacts preferentially with the closed, inactivated state. The reason for these differential interactions is not known. Zamponi and French (1993) recently attempted to dissect these two modes of action on single BTX-modified cardiac Na^+ channels. These authors found that phenol and diethylamine block closed and open channels, respectively. They suggested that neutral lidocaine (equivalent to phenol) is the active form in binding with closed BTX-modified cardiac Na^+ channels, whereas charged lidocaine (equivalent to diethylamine) is the active form in binding with open channels; this hypothesis is comparable with that of Mocyzdlowski et al. (1986) for single BTX-modified muscle Na^+ channels. The latter authors discovered that procaine can block both open and closed channels in lipid planar bilayers, a phenomenon similar to that found for tetracaine (D. Bell and G. K. Wang, unpublished data). The structural basis for the behavior of procaine and tetracaine as dual blockers remains unclear.

In this study, we examined the effects of tetracaine on macroscopic BTX-modified Na^+ currents in GH_3 cells to verify that the neutral form is an active form in interacting with closed channels. Tetracaine contains the entire backbone structure of benzocaine (minus two hydrogen atoms) as well as a typical tertiary amine component found in most

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Address reprint requests to Dr. G. K. Wang, Department of Anesthesia, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115. Tel.: 617-732-6879; Fax: 617-732-6927.

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clinically used LAs (see structure 1, below). With a tertiary amine, tetracaine can be positively charged when it is protonated. The ratio of the charged to the neutral form of tetracaine depends on the pH of the solution. In addition, a quaternary derivative of tetracaine and a homolog lacking the 4-amino functional group on the phenyl ring were included in this study. Our results lead us to conclude that charged tetracaine is an active form that interacts strongly with inactivated BTX-modified Na^+ channels in GH_3 cells. Furthermore, the 4-amino functional group attached to the phenyl ring of tetracaine appears to be critical for the potency of tetracaine and for the enhancement of inactivation in BTX-modified Na^+ channels.

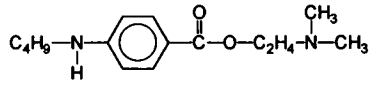
MATERIALS AND METHODS

Chemicals

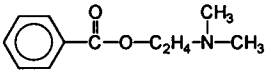
Tetracaine (pK_a = 8.5) was obtained from Sigma Chemical Co. (St. Louis, MO). A tetracaine homolog, 2-(dimethylamino) ethyl benzoate, was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT). Tetracaine and its homolog were dissolved in dimethylsulfoxide (DMSO) at 100–500 mM and maintained as stock solutions at 4°C. BTX was provided by Dr. John Daly (National Institutes of Health, Bethesda, MD) and was dissolved in DMSO at 0.5 mM.

Organic synthesis of *N*-butyl tetracaine QA

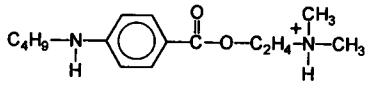
A 1:2 molar ratio of tetracaine base and 1-bromobutane (Aldrich, Milwaukee, WI) was mixed in 15 ml of absolute ethanol and refluxed at 85°C for 30 h. The reaction was stopped and excess ethanol was evaporated. The jelly-like residue was washed several times with warm hexane to remove the remaining starting reactants, and the product was dried under a vacuum. The yield for *N*-butyl tetracaine QA was ~90%, and the product was >97% pure as judged with the thin layer chromatography system. *N*-Butyl tetracaine QA was dissolved in DMSO at 100 mM as a stock solution. The chemical structures of tetracaine and its derivatives are shown below.



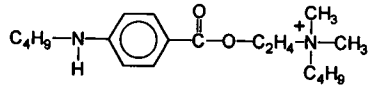
Tetracaine base



2-(Dimethylamino) ethyl benzoate



Charged tetracaine



N-butyl tetracaine QA

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 1× glutamine as described by Cota and Armstrong (1989). The whole-cell variant of the patch-clamp method (Hamill et al., 1981) was used to measure Na^+ currents in GH_3 cells. Two external solutions were used: 0-mM-Na^+ solution, which contained (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-mM-Na^+ solution, which contained (in mM) 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. In some experiments, the buffer of 10 mM HEPES at pH 7.2 was replaced with 10 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.2 titrated with TMA-OH. The junction potential of electrodes was nulled before seal formation. Liquid junction potentials of 3.5 and 1.6 mV for the 130-mM-Na^+ and 0-mM-Na^+ external solutions (Wang and Wang, 1994), respectively, 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. Because of their fast kinetics, normal Na^+ channels were not visible under our voltage-clamp conditions with the slow time base. Drugs were applied to cells via a series of narrow-bored capillary tubes positioned within 200 μm of the cell. The holding potential was set at −100 mV. Voltage-ramp protocols were created with pClamp software (Axon Instruments, Inc., Foster City, CA). Leak and capacitance were subtracted by a homemade device. All experiments were performed at room temperature (23 ± 2°C). At the end of the experiments, the drift in the junction potential was generally <2 mV. Results of analyses are presented as mean ± SE.

RESULTS

Effects of tetracaine on steady-state inactivation of BTX-modified Na^+ channels

We have previously characterized the steady-state inactivation (h_∞) of unmodified and BTX-modified Na^+ channels in GH_3 cells (Wang and Wang, 1992a). Our results showed that a significant number of BTX-modified Na^+ channels were inactivated near −70 mV (e.g., Fig. 1 *A*; Fig. 2, ○). To determine whether tetracaine interacts with this closed state, we measured the h_∞ parameter with various prepulse potentials in the absence and presence of 30 μM tetracaine. Fig. 1 shows the current traces at +50 mV before and after 30 μM tetracaine treatment with a prepulse of −170 mV (Fig. 1 *B*) and −70 mV (Fig. 1 *C*) for 5 s. With the −170-mV prepulse, ~50% of the current was inhibited by 30 μM tetracaine; this result suggested that tetracaine may interact with the closed (possibly resting) state of BTX-modified Na^+ channels almost as effectively as with open channels at +50 mV. There was no apparent time-dependent block of Na^+ currents by tetracaine, a finding distinctly different from those obtained with open-channel blockers such as cocaine and bupivacaine (Wang and Wang, 1992b). These open-channel blockers elicited a significant time-dependent block of Na^+ currents under the same pulse protocol. This result (Figs. 1 *B* and 2) is consistent with the notion that the degree of resting tonic block by tetracaine at voltages between −130 and −170 mV is substantial. Alternatively, tetracaine could elicit an extremely fast block of Na^+ currents that reaches its completion

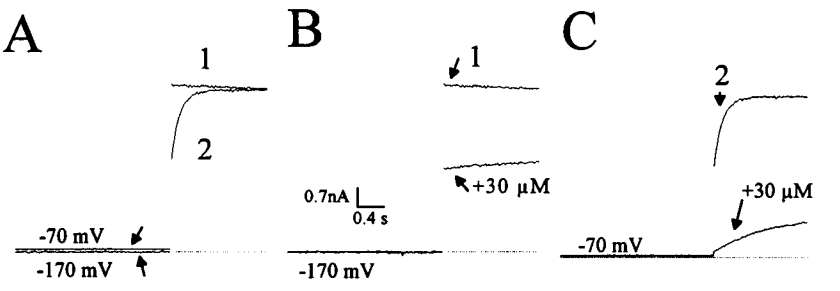


FIGURE 1 BTX-modified Na^+ currents before and after external application of 30 μM tetracaine. (*A*) Control current traces at +50 mV were recorded with prepulses of −170 mV (trace 1) and −70 mV (trace 2). Notice that trace 2 in (*A*) consisted of fast and slow rising phases. Tetracaine was then applied externally at a concentration of 30 μM for 2–3 min and the current traces at +50 mV were again recorded and superimposed with the drug-free record with a −170-mV prepulse (trace 1, *B*) or with a −70-mV prepulse (trace 2, *C*).

within the first 15 ms of depolarization. This possibility is less likely because an on-rate constant of tetracaine reaching about $>1.0 \times 10^7$ M^{-1} s^{-1} would be required. Most LAs characterized as open-channel blockers have an on-rate constant of 60 1.0×10^5 M^{-1} s^{-1} (e.g., Wang, 1988).

With the −70-mV prepulse for 5 s, more than 80% of Na^+ currents were initially blocked when tested at +50 mV (Fig. 1 *C*); the currents then rose slowly and eventually reached steady state. Fig. 2 shows the h_∞ curve at various prepulse voltages. The amplitudes of the initial fast component of Na^+ currents were measured at +50 mV with each prepulse potential and were normalized with respect to the current amplitude measured with a prepulse of −170 mV. Clearly, the block of tetracaine is maximal at −70 mV (Fig. 2, ●), indicating that inactivated channels have the highest affinity toward tetracaine. The potency of tetracaine was directly measured as described next.

Concentration-dependent block of BTX-modified Na^+ channels by tetracaine

The dose-response relation for tetracaine was determined by a method similar to that used previously for benzocaine (Wang and Wang, 1994). Briefly, the inhibition of BTX-modified Na^+ currents at a test pulse of +50 mV was measured in the presence of tetracaine at concentrations ranging from 1 to 300 μM. With a 5-s, −70-mV prepulse, Na^+ currents activated by the test pulse again showed a biphasic pattern (Fig. 3). As the tetracaine concentration was increased, the fast rising phase, which presumably represented the population of resting Na^+ channels free of bound drug, was progressively reduced. The initial inhibition of Na^+ currents was measured, normalized, and plotted against the tetracaine concentration. This dose-response curve yielded an apparent inhibitory concentration (IC_{50}) of 5.2 ± 0.2 μM (n = 5) at −70 mV (Fig. 4, ○). The kinetics of the second rising phase, which represented the reopening of the inactivated channels, were also concentration dependent. The current rose more slowly to reach steady state as the tetracaine concentration increased. The good fit of the data from −70 mV (Fig. 4, ○; Hill coefficient 0.98 ± 0.02) with the Langmuir isotherm demonstrates that one tetracaine molecule blocks one BTX-modified Na^+ channel at this voltage.

With a prepulse of −170 mV, no second rising phase was apparent at concentrations between 3 and 300 μM, nor was a time-dependent block observed at the test pulse (Fig. 3 *B*). The dose-response relation suggested that the tonic block at −170 mV is similar to the block of open channels at +50 mV, a finding predicted by the h_∞ measurement. The dose-response curve yielded an IC_{50} value of 39.5 ± 2.5 μM (n = 5; Fig. 4, ●) for tetracaine block at +50 mV, with a Hill coefficient of 0.94 ± 0.05; this result again suggested that one tetracaine molecule probably blocks one channel. At this voltage, however, a fraction of the open channels could become inactivated and subsequently stabilized by tetracaine binding. Some of the open channels were probably blocked by tetracaine in their open conformation. Hence, the IC_{50} might reflect the potency of tetracaine for more than one channel conformation at this voltage.

Concentration-dependent block of BTX-modified Na^+ channels by tetracaine under voltage-ramp conditions

Using a voltage-ramp protocol, we directly characterized the voltage-dependent block of Na^+ currents by tetracaine. BTX-modified Na^+ currents were activated by a ramp protocol from −130 to +50 mV with a ramp of 15- or 60-s duration. The channel activation threshold, although varying from cell to cell, generally occurred around −85 to −70 mV. After reaching threshold voltages, Na^+ currents rose almost linearly. In the presence of tetracaine, the currents generated by the voltage-ramp protocol were inhibited in a concentration-dependent manner (Fig. 5 *A*). The lack of significant differences in current traces with either a 15- or a 60-s ramp duration (data not shown) suggested that a quasi-steady state of block by tetracaine was reached when channels were fully activated (≥ −40 mV). From −50 mV to +50 mV, the block by tetracaine was clearly voltage dependent. The stronger block by tetracaine at −50 mV than at +50 mV was expected from the h_∞ measurement and the dose-response curve determined by the voltage-step method. Below −50 mV, the block appeared to level off (Fig. 5 *B*) and then decreased at the more negative potential. This result may have been due in part to the higher error associated with small currents and in part to the nonsteady-state condition in the voltage-ramp protocol while the channels were at the activation threshold (see Discussion). Despite this drawback, this protocol, with its wide voltage range, is useful in the direct comparison of the potency of tetracaine under various conditions that will be described next.

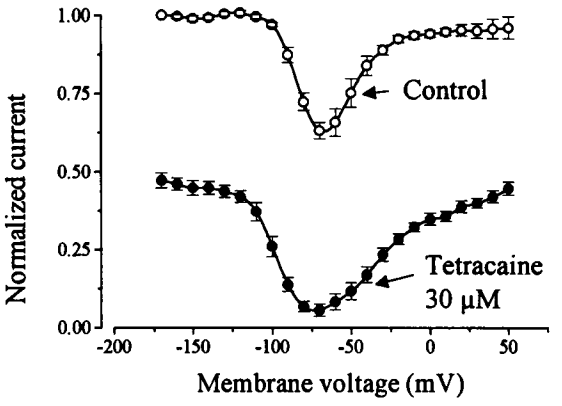


FIGURE 2 Effect of tetracaine on the steady-state inactivation of BTX-modified Na^+ channels. Normalized Na^+ currents were plotted against prepulse voltages. The current amplitudes at the first 15–30 ms of a +50-mV test pulse, with various prepulse voltages of 5 s (as shown in Fig. 1), were measured and normalized against the control-current amplitude with a −170-mV prepulse before external tetracaine application. Data were pooled from five separate cells with (●) or without (○) external 30 μM tetracaine.

pH_i-dependent block of BTX-modified Na^+ channels by tetracaine

Changing the pH of the internal solution (pH_i) alters the intracellular concentration of the charged and neutral forms of tetracaine, which has a pK_a value of 8.5. The neutral form accounts for ~5.3% of the total intracellular tetracaine concentration at pH_i 7.2 and for ~0.5% (a 10-fold reduction) at pH_i 6.2. If only the neutral form of intracellular tetracaine were active in blocking the inactivated BTX-modified Na^+ channel, the potency of tetracaine would decrease at an internal pH of 6.2. Fig. 6 shows that the opposite is the case. Within the range −65–+50 mV, more currents were inhibited by tetracaine at pH_i 6.2 (Fig. 6 *B*). In addition, a stronger rectification of current at 0 to +50 mV, which is typical for open-channel blockers, was evident. This result suggested that the charged form of tetracaine is a dual blocker that behaves as an inactivation enhancer at −50 mV and may also behave as an open-channel blocker when the membrane is depolarized.

Quantitatively, there were only small changes in the protonated tetracaine concentration of the internal solution when the pH_i was lowered from 7.2 to 6.2 (94.7% vs. 99.5%, a <5% increase). It was puzzling, then, to observe that lowering of the pH_i to 6.2 gave rise to such an increase in tetracaine potency (Fig. 6*B*). We will return to this point in the Discussion section.

Antagonistic effects of external Na^+ ions on tetracaine block

External Na^+ ions reduce the potency of open-channel blockers such as QX-314, cocaine, and QA compounds (Wang, 1988; Wang et al., 1991) but have no effect on the potency of the neutral inactivation enhancer benzocaine (Wang and Wang, 1994). It was suggested that electrostatic repulsion between inflowing Na^+ ions and charged LAs might be the cause for the observed reduction in LA affinity (Wang, 1988). To assess this notion, we raised the external Na^+ concentration and measured the blocking effects of tetracaine. Fig. 7, *A* and *B*, shows that Na^+ ions significantly reduced the potency of tetracaine at voltages between −75 mV and +50 mV. It is interesting that very little inhibition of Na^+ currents occurred within the voltage range for channel activation (−85–−65 mV). For example, at −70 mV fewer than 40% of channels were blocked (Fig. 7 *C*). The reason for this phenomenon is not clear, but a similar trend was found in the absence of external Na^+ ions. In any event, reduction of tetracaine potency at voltages near −70 mV by external Na^+ ions is consistent with the ideas that the charged form of tetracaine is responsible for the enhancement of inactivation and that electrostatic repulsion between inflowing Na^+ ions and charged tetracaine is the cause for the decrease in tetracaine affinity. It should be noted here that external Na^+ ions probably also reduced the potency of tetracaine's interaction with Na^+ channels when the membrane was depolarized. At +50 mV, <30% of Na^+ current was blocked in the presence of 130 mM external Na^+ ions (Fig. 7 *B*), whereas >50% was blocked in the absence of external Na^+ ions for the same cell (Fig. 7 *A*).

Quaternary derivative of tetracaine as an inactivation enhancer

Because both types of experiments shown in Figs. 6 and 7 suggested that the charged form of tetracaine is an inactivation enhancer, we synthesized a quaternary derivative of tetracaine (*N*-butyl tetracaine QA) and tested its blocking effect. Like most QA blockers, *N*-butyl tetracaine QA when applied externally inhibited BTX-modified Na^+ currents with much slower kinetics than its tertiary amine counterpart; as a result, steady-state block could not be reached within 2 min. To facilitate the membrane penetration of charged *N*-butyl tetracaine QA, we applied 200 μM externally. Fig. 8 shows the slow action of this drug. It took more than 10 min to block most of the Na^+ current; the block of Na^+ currents by 200 μM *N*-butyl tetracaine QA for 10 min, however, was nearly equivalent to that by 100–300 μM tetracaine (Fig. 5 *A*). The washout of the drug was extremely slow and only partially reversible. This observation suggested that most of *N*-butyl tetracaine QA was trapped within GH_3 cells after prolonged incubation, as has been noted for other amphipathic QA compounds tested in this system. Treatment of *N*-butyl tetracaine QA also elicited a strong block of Na^+ currents at the depolarized potentials. Current rectification was evident at voltages between −10 and +50 mV after 5 min of QA treatment. This rectification was probably due to the tetracaine QA binding site located within the membrane electric field.

Effects of the tetracaine homolog 2-(dimethylamino) ethyl benzoate

Previous studies had suggested that the four-amino group in the structure of benzocaine was important for enhancing the

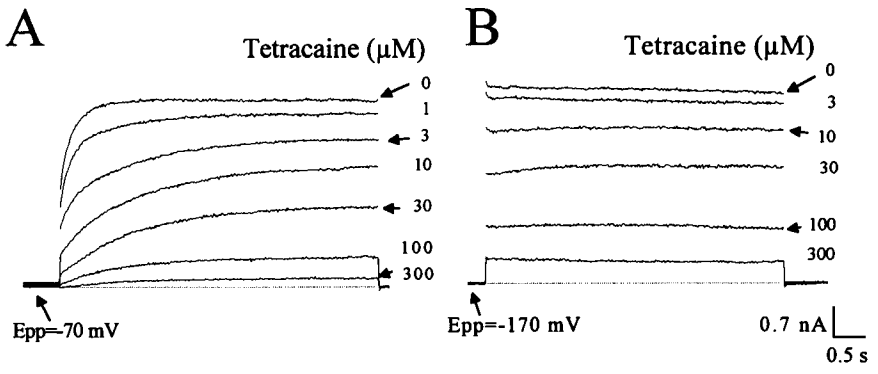


FIGURE 3 Inhibition of BTX-modified Na^+ currents by various concentrations of tetracaine. Currents were activated by a test pulse of +50 mV with a prepulse (Epp) of −70 mV (*A*) or −170 mV (*B*) for 5 s. Current traces were superimposed before and after drug application for comparison. Tetracaine (at the concentration indicated) was applied for 2–3 min while the cell was held at −100 mV. With an Epp of −70 mV, a biphasic rising pattern of Na^+ current was apparent (*A*), whereas only a rapid rising phase, too fast to be recorded, was present with an Epp of −170 mV (*B*). Both (*A*) and (*B*) were recorded from the same cell.

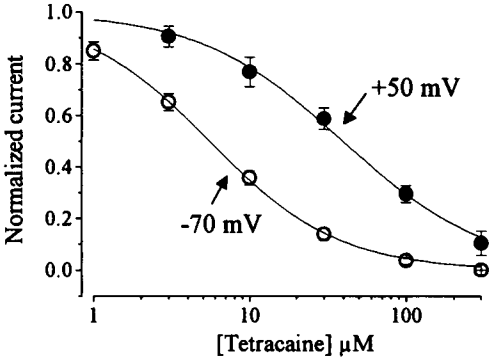


FIGURE 4 Dose-response curve for tetracaine block of BTX-modified Na^+ currents. The amplitudes of the fast rising phase of BTX-modified Na^+ currents (shown as in Fig. 3 *A*) at various tetracaine concentrations were measured, normalized with respect to the control-current amplitude, and plotted against the tetracaine concentration (○, n = 5). The solid line represents the best fit of the data with a Hill coefficient of 0.98 ± 0.02. The steady-state currents maintained at the end of the test pulse of +50 mV were analyzed (in a manner similar to that described in Fig. 3 *B*) with a prepulse of −170 mV and were plotted against the tetracaine concentration (●, n = 5). The Hill coefficient was 0.94 ± 0.05 for this set of data.

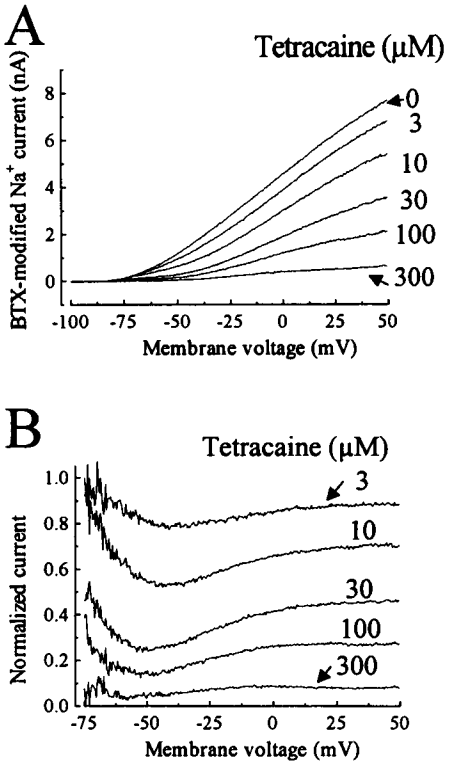


FIGURE 5 Voltage dependence of tetracaine block in BTX-modified Na^+ channels under voltage-ramp conditions. Na^+ currents (*A*) were activated by a voltage-ramp protocol from −130 to +50 mV linearly for 15 s. Current traces shown from −100 to +50 mV were superimposed with or without tetracaine. Tetracaine (at the concentrations indicated) was applied externally for 2–3 min while the cell was held at −100 mV. The amplitudes of Na^+ currents in (*A*) at various tetracaine concentrations at each voltage were measured, normalized with respect to the control-current amplitude, and plotted against the voltage (*B*). Because of the relatively small current amplitude below −50 mV, a higher level of noise was produced after normalization. Results were similar in six other cells.

inactivation of Na^+ channels (Wang and Wang, 1994). To assess this possibility, we tested a structure-related homolog of tetracaine under voltage-ramp conditions. This homolog lacks the butylated four-amino group in the phenyl ring (see structure. Fig. 9 shows that 1.5 mM or 5 mM 2-(diethylamino) ethyl benzoate was much less active than 100 μM tetracaine in blocking Na^+ currents. There may have been some interactions of the inactivated Na^+ channels and 1.5 mM 2-(dimthylamino) ethyl benzoate, given that more block was found near −50 mV than other voltages. Nevertheless, the IC_{50} of this block was about two orders of magnitude larger than that of tetracaine (~1.5 mM vs. ≤10 μM at −50 mV). This result suggested that the [—NH(—C_4H_9)] functional group attached to the phenyl ring of tetracaine increases the potency by about two orders of magnitude at −50 mV.

DISCUSSION

The main quest of this investigation was to confirm that only the neutral form of tetracaine enhances the inactivation of BTX-modified Na^+ channels. Our results show that, contrary to this hypothesis, the charged form of tetracaine is mostly responsible for enhancing inactivation at pH 7.2. The dose-response curve indicates that there is one single binding site for tetracaine in the inactivated channel. This binding site is probably located within the Na^+ permeation pathway. On the basis of structure-activity studies, we propose that the four-amino group attached to the phenyl ring of tetracaine is important for binding as well as for promoting the inactivation

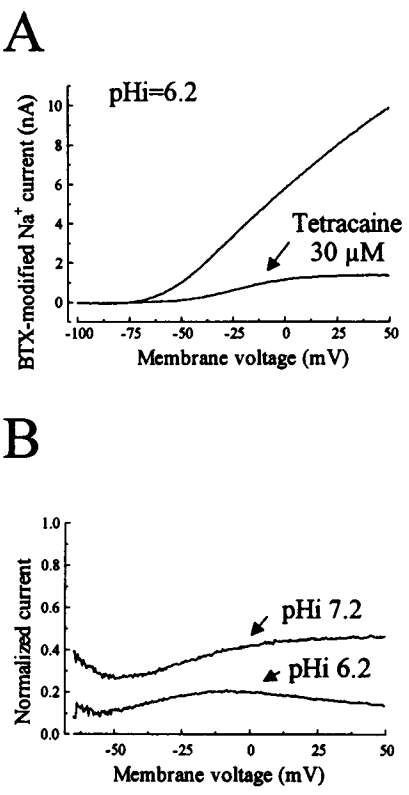


FIGURE 6 Dependence of tetracaine block on pH_i in BTX-modified Na^+ channels under voltage-ramp conditions. Tetracaine at a concentration of 30 μM was applied externally while the cell was held at -100 mV (*A*). The intracellular pH_i was assumed to be 6.2, the same as the pH value for the pipette solution (see Materials and Methods). The amplitudes of Na^+ currents in (*A*) at each voltage were measured, normalized with respect to the control-current amplitude, and plotted against the voltage (*B*). Results were similar in four other cells. The normalized current at pH_i 7.2 was taken from Fig. 5 *B*, which was also treated with 30 μM tetracaine. Because this cell with a pH_i of 6.2 was activated near -70 mV, normalization could not be performed below -65 mV.

of BTX-modified Na^+ channels. In the remainder of this article, we will discuss in detail how these conclusions are derived.

Is the neutral form of tetracaine the only active form?

Several lines of evidence demonstrate that the neutral form of tetracaine is not the primary active form for the blocking action of tetracaine. First, lowering of the internal pH from 7.2 to 6.2, which decreases 10-fold the concentration of the neutral form of intracellular tetracaine, does not reduce its blocking potency. Instead, inactivation is further enhanced by lowering of the intracellular pH, a result indicating that the charged form is active. Second, raising of the external Na^+ concentration significantly reduces the potency of tetracaine in enhancing inactivation. This result would not be expected if the neutral form were the primary active form. Third, the quaternary derivative of tetracaine, *N*-butyl tetracaine QA, is as potent as tetracaine itself in enhancing inactivation. Because *N*-butyl tetracaine QA is permanently charged, there is no reason to believe that charged tetracaine is not active.

Together, these results are inconsistent with the hypothesis that the neutral form of tetracaine is the main active form. However, is it possible that the neutral form is active, although with a lesser affinity than that of the charged form? Our data do not allow us to address this question. Given that benzocaine is a neutral inactivation enhancer, it is reasonable to assume that the neutral form of tetracaine is active. The persistent tonic block of Na^+ channels at a relatively negative potential of <-130 mV suggests that neutral tetracaine can probably reach its binding site when the channel is closed. However, bound neutral tetracaine can easily be protonated by external hydrogen ions (Schwarz et al., 1977). To address the potency of neutral tetracaine will require systematic alteration of internal and external pH. In any event, our results clearly show that charged tetracaine is active, even more so than its neutral counterpart. It is noteworthy that there is a disproportional increase of tetracaine potency when the pH_i was lowered from 7.2 to 6.2 (an increase of less than 5% in the charged form). This phenomenon, however, can be explained if we assume that the pK_a value for tetracaine near its binding site is less than the aqueous value of 8.5. This assumption is likely to be true, given that the measured pK_a of another LA, cocaine, near its binding site in single BTX-modified Na^+ channels is ~7.1 or 1.4 units lower than its aqueous value of 8.5 (Nettleton and Wang, 1990).

Is the tetracaine binding site within the permeation pathway?

The conclusion that the charged form of tetracaine can enhance inactivation suggests that tetracaine binding involves a hydrophilic binding domain encompassing the tertiary amine component. The receptor for this putative hydrophilic binding domain may be located within the Na^+ permeation pathway, given that the external Na^+ ions reduce the binding affinity of LAs significantly. As described in this report and in other studies with and without BTX presence (Cahalan and Almers, 1979; Barber et al., 1992; Zamponi et al., 1993b), Na^+ ions can reduce the potency of charged LAs, probably through electrostatic repulsion. If the reduction of LA potency is attributable to conformational changes at the LA binding site, then one would expect to observe a similar reduction in potency with a neutral LA. No such effect was found; benzocaine, a neutral inactivation enhancer, was not repulsed by external Na^+ ions (Wang and Wang, 1994). It is interesting that, near the channel activation threshold (i.e., -85 to -60 mV), external Na^+ ions are extremely effective in "knocking out" bound tetracaine. This result may be explained by the following. 1) When external Na^+ ions enter the membrane electric field within the Na^+ pore, they are forcefully driven in by the negative electric potential, and the inflowing Na^+ ions then repulse the bound charged tetracaine. 2) The bound charged tetracaine is also within the electric field and is forcefully driven out of the pore by this

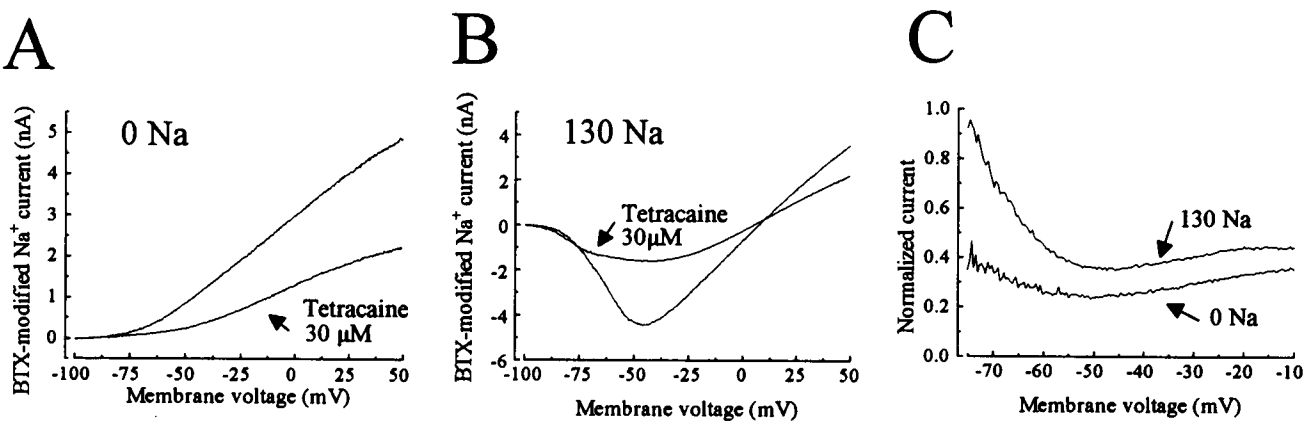


FIGURE 7 Effect of external Na^+ ions on tetracaine block in BTX-modified Na^+ channels. Tetracaine at a concentration of 30 μM was applied externally in solutions containing no Na^+ ions (*A*) or 130 mM Na^+ ions (*B*). Both (*A*) and (*B*) were recorded from the same cell, with use of the standard voltage-ramp protocol. Results were similar in five other cells. For an unknown reason, the reversal potential of Na^+ currents (*B*) was generally right-shifted in GH_3 cells (~6 vs. 0 mV; Wang and Wang, 1994). The amplitudes of Na^+ currents in (*A*) and (*B*) at each voltage were measured, normalized in relation to the control-current amplitude, and plotted against the voltage (*C*). Because of the small current amplitude near the reversal potential in (*B*), normalization was performed only from −75 to −10 mV.

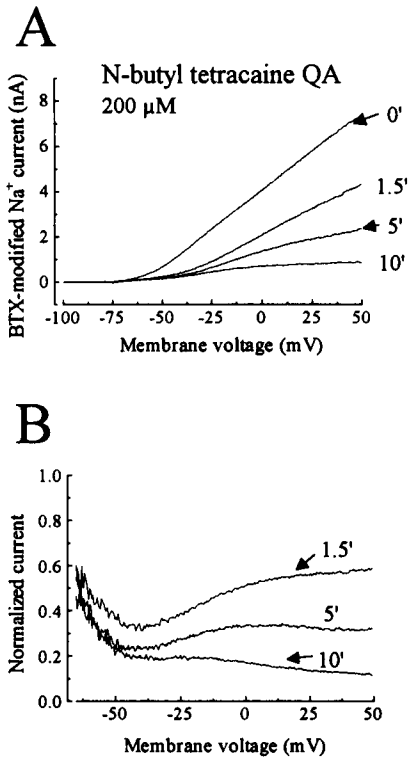


FIGURE 8 Effect of *N*-butyl tetracaine QA on BTX-modified Na^+ currents under voltage-ramp conditions. *N*-Butyl tetracaine QA at a concentration of 200 μM was applied externally for the periods indicated in (*A*). Currents were superimposed for comparison. The external solution contained no Na^+ ions. The amplitudes of Na^+ currents in (*A*) at each voltage were measured, normalized in relation to the control-current amplitude, and plotted against the voltage (*B*). Similar results were found in four other cells.

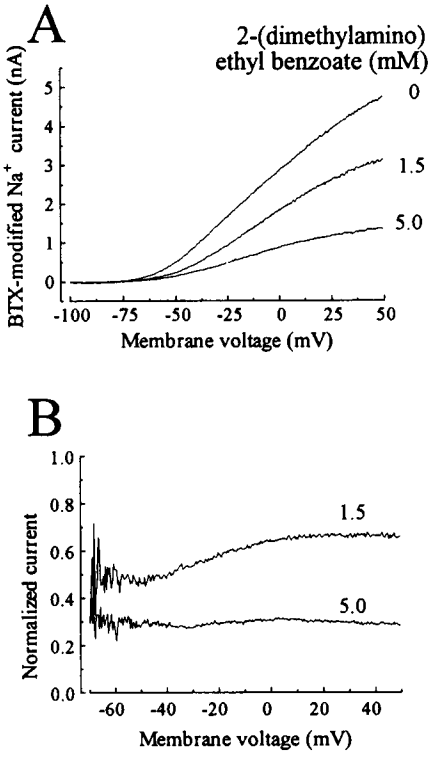


FIGURE 9 Effect of 2-(dimethylamino) ethyl benzoate on BTX-modified Na^+ currents. 2-(Dimethylamino) ethyl benzoate was applied externally at 1.5 or 5 mM in the solution without external Na^+ ions for 2–3 min (*A*). Currents were superimposed for comparison. The amplitude of Na^+ currents in (*A*) at each voltage were measured, normalized in relation to the control-current amplitude, and plotted against the voltage (*B*). The potency of 2--(dimethylamino) ethyl benzoate was about two orders of magnitude lower than that of tetracaine (Fig. 5 *A*). Results were similar in four other cells.

negative potential. In other words, at −85–−60 mV, the membrane potential favors the entry of external Na^+ ions into the pore and the exit of bound charged tetracaine.

It is noteworthy that steady-state inactivation with and without tetracaine (Fig. 2) occurs maximally at −70 mV. We interpret this result, along with the dose-response curve measured by the voltage-step method at −70 mV (Fig. 4), to mean that inactivated BTX-modified Na^+ channels have the

highest affinity for tetracaine. Why, then, is the potency of tetracaine weakened at voltages between −85 and −60 mV, as measured by the voltage-ramp protocol (Fig. 5 *B*)? The reason for this discrepancy is probably related to the blocking kinetics of tetracaine. We found that at −70 mV it took ~2 s for the block of tetracaine (at a concentration of 30 μM) to reach completion. Hence, in the measurement of h_∞, the prepulse duration was set for 5 s. However, during the voltage-ramp protocol the total duration was set for 15 s from −130 mV to +50 mV. It is likely that even a quasi-steady state cannot be achieved at −70 mV under these conditions (i.e., 12 mV/s of ramp speed). In addition, if the activation gate, when closed, prevented the charged form of tetracaine from entering the pore (and the tetracaine binding site was assumed to be within the permeation pathway), such a phenomenon would be expected because the activation gate fluctuated between open and closed states at voltages of <−40 mV, and the access of charged tetracaine might be limited. It is likely that the charged form of tetracaine has greater access to open channels than to their closed counterparts. As channel activation reaches its completion around −40 mV (Wang and Wang, 1994) so does the block of tetracaine reach its quasi-steady state under the voltage-ramp protocol (Fig. 5 *B*). This explanation is also consistent with the observation shown in Fig. 8 *B*. As the Na^+ channel reaches its activation threshold, *N*-butyl tetracaine QA can access its receptor when the channel is in its open form. The availability of the open form becomes a rate-limiting step for the charged drug during the voltage-ramp protocol between −75 and −50 mV, despite the fact that the internal concentration of *N*-butyl tetracaine QA may continue to increase after 5 min of treatment.

Are there overlapping binding sites for the inactivation enhancer and the open-channel blocker?

The binding site for the inactivation enhancer probably lies within the Na^+ permeation pathway, as discussed above. Open-channel blockers such as cocaine, bupivacaine, and amphipathic QA compounds are also likely to be within the pore (e.g., Wang et al., 1991). Voltage-dependent blocking characteristics indicate that the binding site for these open-channel blockers is located about halfway into the electric field. Furthermore, external Na^+ ions reduce the potency of open-channel blockers, again probably through electrostatic effects. Given that the voltage-dependent blocking characteristics of inactivation enhancers parallel the h_∞ curve, it is not possible to determine directly the relative location of their binding site within the electric field. Nonetheless, the available evidence shows that tetracaine is a dual blocker that binds preferentially with the inactivated state but can also block the open channel. For example, at pH 6.2, the strong rectification of the tetracaine block at the 0- to 50-mV range (Fig. 6 *B*) indicates that positively charged tetracaine is driven into the open-channel pore when the membrane potential is depolarized. This phenomenon is commonly documented for open-channel blockers. A similar rectification was found for the QA derivative of tetracaine within this voltage range (Fig. 8 *B*). Finally, in bilayer experiments tetracaine induced long-lasting closures and reduced the single-channel conductance of rabbit muscle BTX-modified Na^+ channels (particularly at more depolarized potentials, data not shown). This observation is consistent with those in previously reported bilayer studies of procaine (Moczydlowski et al., 1986). Unfortunately, our initial attempts to study the detailed action of tetracaine in single BTX-modified muscle Na^+ channels in bilayers were hampered by the frequent disappearance of single Na^+ channels in the presence of tetracaine. Furthermore, the induced closures by tetracaine were relatively long and highly variable from channel to channel (average mean closed time at 300 μM tetracaine ranging from 1 to 8 s, which made kinetic analyses difficult). These difficulties were reported previously for benzocaine and procaine in the bilayer system (Moczydlowski et al., 1986).

To explain the complicated action of tetracaine on BTX-modified Na^+ channels in GH_3 cells, we apply the concepts of the modulated receptor hypothesis (Hille, 1977, 1992b) and suggest that overlapping binding sites for inactivation enhancers and open- channel blockers are present for all LA drugs and QA compounds. In the absence of evidence for two clearly separate tetracaine binding sites in BTX-modified Na^+ channels, as determined by the Hill coefficient of ~1 (Fig. 4), the following simple kinetic scheme with a single binding site can be used to describe our results.

$$\begin{array}{ccc} & C & \rightleftharpoons & O \\ * & \downarrow\uparrow & * & \downarrow\uparrow \\ & C^* & & O^* \end{array} \qquad \text{(Scheme 1)}$$

where O is the open state, C is the closed state (which includes the resting and inactivated states), and * is tetracaine. The C ↔ O ↔ O* pathway is proposed for open-channel blockers (type 1 LAs) and the O ↔ C ↔ C* pathway for inactivation enhancers (type 2 LAs). Hence, the block of tetracaine and its mode of action are state dependent. At −70 mV, tetracaine binds to the inactivated state with a high affinity and "stabilizes" such a conformation upon binding. Less binding occurs between tetracaine and the resting closed channels or open channels. At +50 mV, inactivated channels are forced to open (Wang and Wang, 1992a) but tetracaine may still interact with the O state as an open-channel blocker. In the presence of tetracaine, a mixture of the C* state and the O* state probably coexists at +50 mV. It is likely that the O ↔ O* pathway at +50 mV elicits a fast open-channel block by reducing single-channel conductance, as in bilayers. Such a pathway presumably competes with the O ↔ C ↔ C* pathway in a mutually exclusive manner; i.e., one channel can bind only one tetracaine molecule at a time. Separation of these two states cannot be resolved at the macroscopic current level, nor can the pathway of C* ↔ O* be ascertained.

Recently, Zamponi and French (1993) studied the effects of diethylamine and phenol on single BTX-modified cardiac

Na^+ channels. They suggested that there are two separate binding sites: one for the fast block of the open channel ($C \leftrightarrow O \leftrightarrow O^*$ pathway) and another for the slow block of the closed inactivated channel ($O \leftrightarrow C \leftrightarrow C^*$ pathway) (but see Gingrich et al. (1993) for a one-site theory regarding normal cardiac Na^+ channels). In particular, these authors envisioned the binding of the neutral form of the drug to a site located in a hydrophobic environment close to the receptor near the inactivation gate. Although our results showed that the presence of a neutral form of drug is not a prerequisite for tetracaine binding with the inactivated channel and that the tetracaine binding site is within the Na^+ permeation pathway, we were unable to exclude the two-site theory. It may prove difficult to eliminate such a possibility because the effects of the drug by specific and nonspecific binding sites for neutral anesthetics, particularly at high concentrations, tend to be superimposed (for details see Elliott and Haydon, 1989).

Is the common four-amino group in inactivation enhancers important for binding?

To address this question, it is necessary to examine the structure of open-channel blockers and inactivation enhancers. We have noticed that all inactivation enhancers, such as benzocaine, tetracaine, and procaine, contain a common four-amino group (a three-amino group for tricaine) on the phenyl ring, whereas the open-channel blockers such as cocaine, bupivacaine, and amphipathic QA compounds, do not. An exception to this rule is procainamide, which acts as an open-channel blocker (Zamponi et al., 1993a). However, this compound has an amide bond (instead of an ester bond) linked with the phenyl ring. The planar amide bond may position the phenyl ring at an angle that diminishes the interactions of the four-amino group with the Na^+ channel. In addition, we omit the discussion of lidocaine as an inactivation enhancer because such a phenomenon is so far found only in cardiac Na^+ channels (Zamponi et al., 1993c). We have previously reported that dimethylation of the four-amino group of benzocaine diminishes its ability to enhance inactivation effectively. Given that a single alkylation of the four-amino group in tetracaine does not destroy its potency in enhancing inactivation, the preferred binding between the Na^+ channel and the four-amino group of tetracaine probably still takes place. Unfortunately, the exact contribution of the butyl group in tetracaine potency cannot be directly assessed, because we were unable to obtain the compound without the butyl group. Deletion of the 4-butylamino group, however, reduces the potency of tetracaine as an inactivation enhancer by ~100-fold (IC_{50} = ~1.5 vs. $\leq$ 10 μM at −50 mV). This result is consistent with the idea that drugs with a four-amino group (or 4-butylamino group) on the phenyl ring can stabilize the inactivated state much more efficiently than those without such a group. If so, the 4-butylamino group of tetracaine may actually interact (directly or indirectly) with the inactivation gating mechanism, such as stabilizing the inactivation docking receptor and/or the inactivation "ball" when the channel is in its inactivated state. In terms of Scheme 1, we surmise that the interaction with the 4-butylamino group is enhanced (i.e., as an inactivation enhancer) when the BTX-modified Na^+ channel is in its inactivated state. For open channels at the more depolarized voltage, this interaction with the 4-butylamino group of tetracaine is weakened (i.e., as an open-channel blocker), probably as a result of conformational changes at the tetracaine binding site.

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REFERENCES

Barber M. J., D. J. Wendt, C. F. Starmer, and A. O. Grant. 1992. Blockade of cardiac sodium channels: competition between the permeant ion and antiarrhythmic drugs. *J. Clin. Invest.* 90:368–381.

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

Catterall, W. A. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* 20: 15–43.

Cota, G., and C. M. Armstrong. 1989. Sodium channel gating in clonal pituitary cells: the inactivation step is not voltage dependent. *J. Gen. Physiol.* 94:213–232.

Elliott, J. R., and D. A. Haydon. 1989. The actions of neutral anesthetics on ion conductances of nerve membranes. *Biochim. Biophys. Acta.* 988: 257–286.

Gingrich, K. J., Beardsley, D., and Yue, D. T. 1993. Ultra-deep blockade of Na^+ channels by a quaternary ammonium ion: catalysis by a transition-intermediate state? *J. Physiol.* 471:319–341.

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

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Archiv.* 391: 85–100.

Hille, B. 1977. Local anesthetics: hydrophilic and hydrophobic pathways for the drug receptor reaction. *J. Gen. Physiol.* 69:497–515.

Hille, B. 1992a. Modifiers of gating. *In* Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, Massachusetts. 445–471.

Hille, B. 1992b. Mechanisms of block. *In* Ionic channels of excitable membranes. Sinauer Associations, Sunderland, Massachusetts. 390–422.

Huang, L. Y. M., A. Yatani, and A. M. Brown. 1987. The properties of batrachotoxin-modified cardiac Na channels, including state-dependent block by tetrodotoxin. *J. Gen. Physiol.* 90:341–360.

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

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

Mozhayeva, G. N., A. P. Naumov, and B. I. Khodorov. 1986. A study of properties of batrachotoxin modified sodium channels. *Gen. Physiol. Biophys.* 5:17–46.

Nettleton, J., and G. K. Wang. 1990. pH-dependent binding of local anesthetics in single BTX-activated Na^+ channels: cocaine vs. quaternary compounds. *Biophys. J.* 58:95–106.

Schneider, M. F., and J. M. Dubois. 1986. Effects of benzocaine on the kinetics of normal and batrachotoxin-modified Na channels in frog node of Ranvier. *Biophys. J.* 50:523–530.

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

Strichartz, G., R., T. A. Rando, and G. K. Wang. 1987. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* 10:237–267.

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

Wang, G. K., R. Simon, and S.-Y. Wang. 1991. Quaternary ammonium compounds as structural probes of single batrachotoxin-activated Na^+ channels. *J. Gen. Physiol.* 99:1–20.

Wang, G. K., and S.-Y. Wang. 1992a. Inactivation of BTX-modified Na^+ channels in GH_3 cells: characterization and pharmacological modification. *J. Gen. Physiol.* 99:1–20.

Wang, G. K., and S.-Y. Wang. 1992b. Altered stereoselectivity of cocaine and bupivacaine isomers in normal and BTX-modified Na^+ channels. *J. Gen. Physiol.* 100:1003–1020.

Wang, G. K., and S.-Y. Wang. 1994. Binding of benzocaine in batrachotoxin-modified Na^+ channels: state-dependent interactions. *J. Gen. Physiol.* 103:501–518.

Wasserstrom, J. A., K. Liberty, J. Kelly, P. Santucci, and M. Myers. 1993. Modification of cardiac Na^+ channels by batrachotoxin: effects on gating, kinetics, and local anesthetic binding. *Biophys. J.* 65:386–395.

Zamponi, G. W., P. W. Codding, X. Sui, and R. J. French. 1993a. Dual actions of procainamide on batrachotoxin-activated cardiac sodium channels. Open channel block and prevention of inactivation. *Biophys. J.* 65:2324–2334.

Zamponi, G. W., D. D. Doyle, and R. J. French. 1993b. Fast lidocaine block of cardiac and skeletal muscle sodium channels: one site with two routes of access. *Biophys. J.* 64:80–90.

Zamponi, G. W., D. D. Doyle, and R. J. French. 1993c. State-dependent block underlies the tissue specificity of lidocaine action on batrachotoxin-activated cardiac sodium channels. *Biophys. J.* 65:91–100.

Zamponi, G. W., and R. J. French. 1993. Dissecting lidocaine action: diethylamine and phenol mimic separate modes of lidocaine block of sodium channels from heart and skeletal muscle. *Biophys. J.* 65: 2335–2347.

Zubov, A. N., A. P. Naumov, and B. I. Khodorov. 1984. Effects of batrachotoxin on sodium channels of neuroblastoma cells. *Neirofiziologiya.* 16:415–423.