

Local Anesthetics QX 572 and Benzocaine Act at Separate Sites on the Batrachotoxin-activated Sodium Channel

LI-YEN MAE HUANG and GERALD EHRENSTEIN

From the Laboratory of Biophysics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT We have studied the effects of local anesthetics QX 572, which is permanently charged, and benzocaine, which is neutral, on batrachotoxin-activated sodium channels in mouse neuroblastoma N18 cells. The dose-response curves for each drug suggest that QX 572 and benzocaine each act on a single class of binding sites. The dissociation constants are 3.15×10^{-5} M for QX 572 and 2.64×10^{-4} M for benzocaine. Equilibrium and kinetic experiments indicate that both drugs are competitive inhibitors of batrachotoxin. When benzocaine and QX 572 are present with batrachotoxin, they are much more effective at inhibiting Na^+ flux than would be predicted by a one-site model. Our results indicate that QX 572 and benzocaine bind to separate sites, each of which interacts competitively with batrachotoxin.

INTRODUCTION

Local anesthetics have been shown to depress the excitability of nerve fibers by blocking Na^+ channels (Taylor, 1959; Hille, 1966; Narahashi et al., 1968). The extent of inhibition of sodium current is enhanced in some cases by repetitive depolarizing pulses and is reduced in some cases by prolonged hyperpolarization (Strichartz, 1973; Courtney, 1975; Khodorov et al., 1976; Hille, 1977 *b*; Cahalan, 1978; Lipicky et al., 1978; Yeh, 1979). These drugs alter the gating processes of Na^+ current. Benzocaine and a few tertiary and quarternary amine local anesthetics produce a negative voltage shift of sodium inactivation curves (Courtney, 1975; Hille, 1977 *b*; Schwartz et al., 1977). When the sodium inactivation is eliminated with pronase, the voltage-dependent block of many of these drugs is partially or totally abolished (Cahalan, 1978; Yeh, 1979). QX 314, QX 572, and benzocaine have also been shown to immobilize the gating charge of sodium channels in squid axon and frog node (Cahalan and Almers, 1979; Khodorov et al., 1979; Gusel'nikova et al., 1979). These observations led to the suggestion that local anesthetics interact with the inactivation gating mechanism of the Na^+ channels (Courtney, 1975; Hille, 1977 *b*; Yeh, 1979; Cahalan and Almers, 1979).

Because both permanently charged and permanently uncharged local anesthetics shift the inactivation curve in a similar manner, Hille (1977 *b*)

proposed that both types of local anesthetics bind to a single receptor site. In this model, uncharged or lipid-soluble drugs get to the receptor site through the hydrophobic region of the membrane; charged or hydrophilic drugs gain access to the receptor via the lumen of the channel. The apparent differences in the action of charged and uncharged local anesthetics would then be caused by their different rates of action.

Khodorov et al. (1976) suggested that there might be more than one receptor for local anesthetics. Mrose and Ritchie (1978) tested whether a single-receptor-site model could explain the relative effectiveness of mixtures of benzocaine and lidocaine compared with the effectiveness of each drug alone. They first determined concentrations of each of the two drugs that were equipotent in reducing the compound action potential in frog sciatic nerves to a standard amplitude. Then they applied a mixture of half of the equipotent concentration of benzocaine and half of the equipotent concentration of lidocaine. If both drugs act at the same receptor site, and only at that one site, then the mixture should reduce the action potential to the same standard level as either equipotent solution alone. When Mrose and Ritchie applied the mixture, however, they observed a reduction in the amplitude of the action potential that was significantly larger than the reduction caused by either equipotent solution alone. This result contradicts the hypothesis of a single receptor site. It does not rule out the possibility that lidocaine and benzocaine act on the same site, and that, in addition, one of the drugs also acts at another site. Indeed, Mrose and Ritchie suggest this as a plausible explanation.

To determine whether charged and uncharged local anesthetics do, in fact, act at the same site, we have studied the interaction of benzocaine and QX 572, a lidocaine derivative, on batrachotoxin-activated sodium channels in N18 neuroblastoma cells. We chose permanently charged QX 572 and permanently neutral benzocaine for our studies to avoid changes in the degree of ionization of these amine local anesthetics during the experiments, and thus simplified the interpretation of our results. Another reason for choosing QX 572 is that it is relatively lipid soluble, and, therefore, can cross the membrane in a reasonable time. This is important because charged lidocaine derivatives have been shown to act from the internal membrane surface (Frazier et al., 1970; Hille, 1977 *a*).

The Na^+ permeability for different concentrations of the local anesthetics was determined by measuring $^{22}\text{Na}^+$ uptake through batrachotoxin-activated channels. Batrachotoxin (BTX), a toxin produced by the Colombian frog, *Phylllobates aurotaenia*, has been shown to act specifically on Na^+ channels (Albuquerque et al., 1971; Narahashi et al., 1971; Catterall, 1975; Khodorov et al., 1975; Khodorov, 1978) and cause the channels to open persistently. The interaction of externally applied BTX and QX 572 has previously been studied in squid axon by Albuquerque et al. (1973) and in frog node by Khodorov (1978). In the squid experiments, no interaction between BTX and QX 572 was found, whereas in the frog node, there was competitive inhibition. Because QX 572 probably acts only from the internal membrane surface, but was applied externally, the different results may be related to different permeabilities of the drug in the two preparations.

The increase in steady-state $^{22}\text{Na}^+$ influx in the presence of BTX provides an accurate measure of Na^+ permeability through the sodium channels (Catterall, 1977). By using a large number of tissue culture dishes with BTX and various concentrations of one or both local anesthetics, we obtained a set of dose-response curves for each drug alone and for the two drugs together. These curves provide a detailed test as to whether charged or uncharged local anesthetics act at the same site. Preliminary results of this work have been presented (Huang and Ehrenstein, 1980).

MATERIALS AND METHODS

Material

The media and chemicals used were obtained from the following sources: Dulbecco-Vogt modification of Eagle's minimal essential medium (DMEM) and Dulbecco's phosphate-buffered saline from the media unit of the National Institutes of Health, Bethesda, Md.; Fetal calf serum was from Grand Island Biological Co., Grand Island, N. Y.; recrystallized trypsin from Worthington Biochemical Co., Freehold, N. J.; Ouabain from Sigma Chemical Corp., St. Louis, Mo.; Benzocaine from Aldrich Chemical Co., Milwaukee, Wis.; $^{22}\text{NaCl}$ from Amersham/Searle Corp., Arlington Heights, Ill; $[1,2,3\text{-}^3\text{H}]\text{Leucine}$ from New England Nuclear, Boston, Mass. Batrachotoxin was kindly supplied by Dr. J. Daly, Laboratory of Digestive Diseases, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. QX 572 Cl was kindly provided by Dr. B. Takman, Astra Pharmaceutical Product Co., Framingham, Mass.

Cell Culture

Cell lines N18 of mouse neuroblastoma, C1300 were grown as described (Catterall, 1975; Huang et al., 1979). $[^3\text{H}]\text{leucine}$ (0.2 Ci/ml) was added to growth media 1 d before use so that the ^3H in the protein recovered in the experiments could be counted.

Measurement of Influx Rate

Cells were first preincubated with drug and/or toxin at 36°C for 60 min in a sodium-free medium consisting of 135 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4 , and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) (pH 7.4, adjusted with tris(hydroxymethyl)aminomethane [Tris]) and 1 mg/ml of bovine serum albumin. Sixty minutes were long enough to achieve equilibrium, as we found by measurement of sodium uptake as a function of time following addition of drug and toxin. The initial rate of passive $^{22}\text{Na}^+$ influx was measured for 30 s in uptake medium containing the same concentration of toxin and/or drug, 130.0 mM choline chloride, 5.0 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4 , 5.0 mM Ouabain, 50 mM HEPES (pH 7.4; adjusted with Tris) and $1\ \mu\text{Ci/ml}$ $^{22}\text{NaCl}$. The dissociation constant of activation by BTX is 0.4–0.7 μM (Catterall, 1975 and 1977; Huang et al., 1979). 1 μM BTX, which corresponds to 75–90% of the maximum response, was used in most of the experiments. This concentration was chosen because of the low solubility of BTX in water and the limited supply of the toxin. The uptake was terminated by washing the cells four times with 3 ml of 0°C wash medium consisting of 135 mM choline chloride, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5.0 mM HEPES (adjusted pH to 7.4 with Tris base).

Cells were then suspended in 0.6 ml of 0.4 N NaOH and transferred to a scintillation vial containing 1 ml of 1 M Tris-HCl (pH, 7.4) and 10 ml of scintillation mixture

(5.53% [vol/vol], Research Products International Corp. [Elk Grove Village, Ill.] scintillator fluid, 61.14% toluene, and 33.3% Triton X-100). The cell protein was determined by a modification of the Lowry method (Lowry et al., 1951).

RESULTS

Inhibition of QX 572 and Benzocaine

The effect of QX 572 or benzocaine on BTX-activated uptake is given in Fig. 1. QX 572 and benzocaine inhibit 80% of the BTX-dependent Na^+ uptake at about 0.4 and 4 mM, respectively. The dose-response curve (Fig. 1) can be well fit by a modified Michaelis-Menten equation (modified to reflect the inhibitory nature of the drug); $V = V_{\max} [1 - A/(K_i + A)]$, where K_i is the

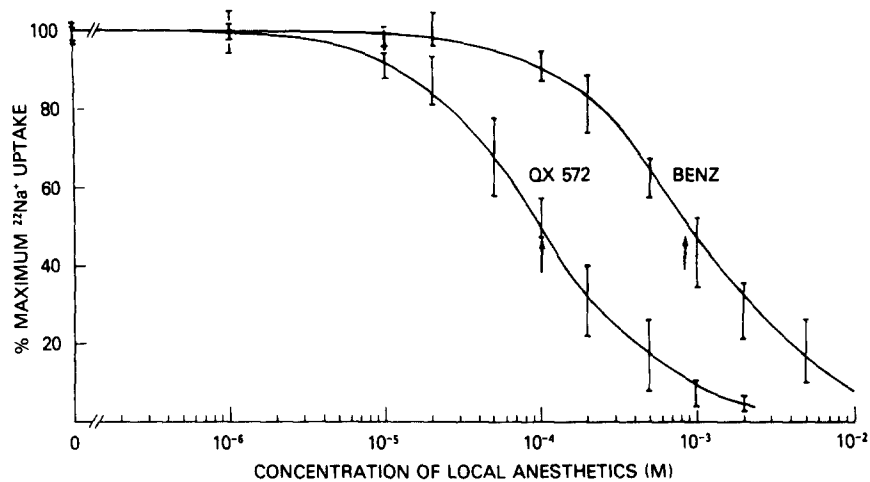


FIGURE 1. Inhibition of BTX-dependent Na^+ uptake by QX 572 and benzocaine. Cells were preincubated with $1 \mu\text{M}$ BTX and the indicated concentrations of local anesthetics for 60 min. $^{22}\text{Na}^+$ uptake was then measured for 30 s in uptake medium containing the same amount of BTX and local anesthetics as the preincubation medium. The solid line is the best fit of the data to the modified Michaelis-Menten equation. The error bars represent standard deviations. The arrows indicate the K_i of QX 572 or benzocaine.

apparent dissociation constant of the inhibitor, A the concentration of local anesthetic, V_{\max} the maximum uptake in the absence of inhibitor, and V the uptake measured at various drug concentrations. The data have a Hill coefficient of ~ 1.0 . These results suggest that benzocaine and QX 572 each act on a single class of binding sites. The apparent dissociation constants are $K_i = 9.0 \pm 2.0 \times 10^{-5}$ M for QX 572 and $K_i = 8.0 \pm 1.5 \times 10^{-4}$ M for benzocaine. Thus, QX 572 is about nine times more potent in blocking Na^+ channels than benzocaine. These results are in general agreement with the work on frog sciatic nerve (Hille, 1977 *b*).

Interaction of QX 572 and Benzocaine with BTX

To determine the inhibition pattern of benzocaine and QX 572, we measured

$^{22}\text{Na}^+$ uptake at various concentrations of BTX in the presence of several different concentrations of QX 572 or benzocaine. The data were plotted on double-reciprocal coordinates (Fig. 2). The experimental results, using an iterative least-square procedure, were fit to the Michaelis-Menten equation: $V = V_{\max} B / (K_a + B)$, where B is BTX concentration, K_a the apparent dissociation constant for BTX. As the local anesthetic concentration increases, the slope of the corresponding reciprocal plot also increases. K_a (reciprocal of the abscissa intercept) increases markedly with increasing drug concentration. In contrast, the maximum level of activation V_{\max} (reciprocal of the ordinate intercept) is little affected. Thus, both QX 572 and benzocaine are competitive inhibitors of BTX activation. A replot of K_a for each reciprocal plot vs. the corresponding drug concentration gives a straight line (Fig. 2, *inset*). The true dissociation constants of QX 572 and benzocaine can be calculated from the abscissa intercepts of the replots. They are $3.15 \pm 0.70 \times 10^{-5}$ M for QX 572 and $2.64 \pm 0.40 \times 10^{-4}$ M for benzocaine. These results suggest that the binding of BTX and the binding of local anesthetics QX 572 or benzocaine are mutually exclusive.

Interaction between QX 572 and Benzocaine

Although QX 572 and benzocaine are both competitive inhibitors of BTX, this does not necessarily imply that QX 572 and benzocaine act on the same site. Competitive inhibition patterns may arise in two ways: (a) the drug binds at the BTX site and then physically prevents the binding of BTX; (b) the drug binds to a site separate from the BTX site. The binding of the drug can then cause either a conformational change in the BTX receptor site or steric hindrance. As a result, BTX can no longer bind. In case b, two drugs could bind to two separate sites and still both be competitive with BTX. To determine the mechanism in the case of QX 572 and benzocaine, we measured the inhibition of BTX-activated Na^+ uptake by QX 572 in the presence of several concentrations of benzocaine or vice versa. If both drugs bind to the same site and only to that site, the apparent dissociation constant K_i for one drug should increase with increasing concentration of the second drug. On the contrary, as shown in Fig. 3, the presence of benzocaine decreases the K_i of QX 572, and the presence of QX 572 decreases the K_i of benzocaine.

DISCUSSION

Batrachotoxin causes as much as a 25-fold increase in the initial rate of Na^+ uptake in N18 mouse neuroblastoma cells (Catterall, 1975). This increase is completely abolished at low concentration of tetrodotoxin—a specific inhibitor of Na^+ channels (Catterall, 1975). On the other hand, neuroblastoma cell lines specifically lacking Na^+ channels (Minna et al., 1971) do not respond to treatment with BTX (Catterall and Nirenberg, 1973; Catterall, 1975 and 1977). Khodorov and co-workers (1975 and 1978) studied the action of BTX in node of Ranvier and observed that BTX-modified Na^+ current developed concurrently with the disappearance of normal Na^+ current. This evidence supports the view that BTX is a specific activator of Na^+ channels and that the increase in the rate of Na uptake reflects the opening of Na^+ channels. In

the discussion to follow, we assume that this specificity of BTX action is correct.

Our equilibrium experiments show that both local anesthetics markedly affect the apparent dissociation constant of BTX and have little effect on the maximum ion flux at 1 μM BTX (Fig. 2). Thus, the binding of QX 572 or benzocaine and the binding of BTX are mutually exclusive.

Our results with QX 572 agree with the finding of Khodorov (1978) that

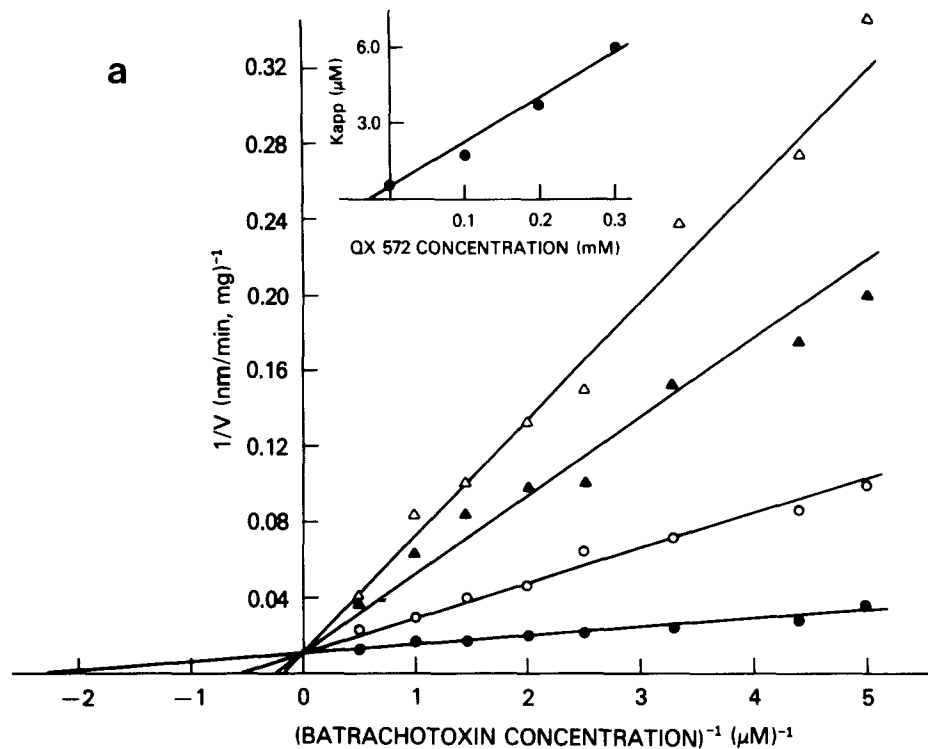
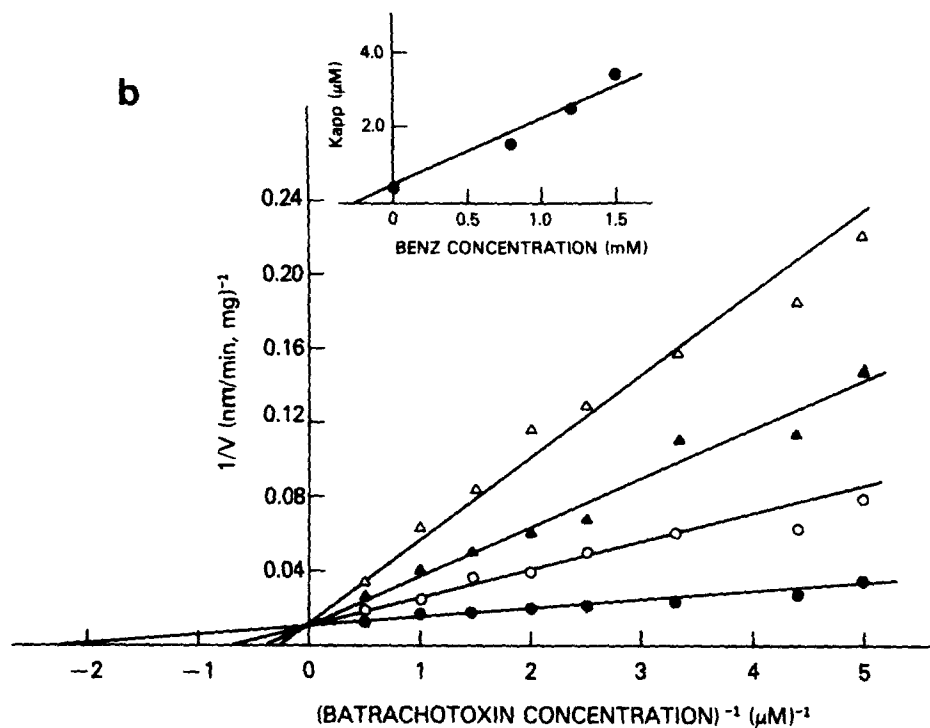


FIGURE 2. Double reciprocal plot describing the interaction of BTX and the local anesthetics. (a) The cells were preincubated with the indicated concentrations of BTX containing 0 (●), 0.1 mM (○), 0.2 mM (▲), 0.3 mM (△) QX 572 for 60 min. $^{22}\text{Na}^+$ uptake was then measured. The solid line is the least square fit to the equation $V = V_{\text{max}} \cdot B / (K_a + B)$, where V is the uptake and V_{max} is assumed to be constant. The K_a 's obtained at different QX 572 concentrations are replotted as a function of QX 572 concentration (*inset*). The intercept on the abscissa gives the true dissociation constant for QX 572. (b) Same experiment as in a with 0 (●), 0.8 mM (○), 1.2 mM (▲), and 1.5 mM (△) benzocaine.

BTX antagonizes the blocking action of this drug. Our results with benzocaine, however, do not agree with Khodorov's observation that the blocking action of benzocaine is not affected by BTX in frog node (Khodorov, 1978). Although the source of this discrepancy is uncertain, a closer look at Khodorov's data does reveal a small interaction between benzocaine and BTX. When 3 mM benzocaine was applied to the frog node, normal Na^+ current was reduced

71%, but BTX-activated Na^+ current was reduced only 58% (Khodorov, 1978). Thus, Khodorov's data is consistent with benzocaine having a small effect on BTX binding in the frog node. As we have shown in this paper, benzocaine is a much less potent inhibitor than QX 572. It is possible that the concentration of benzocaine used in frog node is either not high enough to completely block normal Na^+ current, or is not as effective as QX 572 in displacing BTX. Negulyaev and Nosyreva (1979) have shown that in the frog node the apparent dissociation constant of benzocaine is larger for aconitine-activated Na^+ channels than for normal Na^+ channels. Aconitine and BTX have a common binding site (Catterall, 1977) and these two alkaloid toxins affect activation, inactivation, and selectivity of Na^+ channels in a very similar



way (Schmidt and Schmidt, 1974; Mozhayeva et al., 1977). Thus, it is consistent that benzocaine interacts competitively with BTX as well as with aconitine.

We have shown in Fig. 1 that QX 572 and benzocaine each reduces the BTX-activated Na^+ flux. Although the experimental data of Fig. 1 are not sufficiently accurate to rule out the possibility that each local anesthetic binds to multiple sites, a better fit is obtained under the assumption that each local anesthetic binds to a single site.

We have shown in Fig. 3 that the apparent dissociation constant of either of these local anesthetics decreases when a fixed concentration of the other

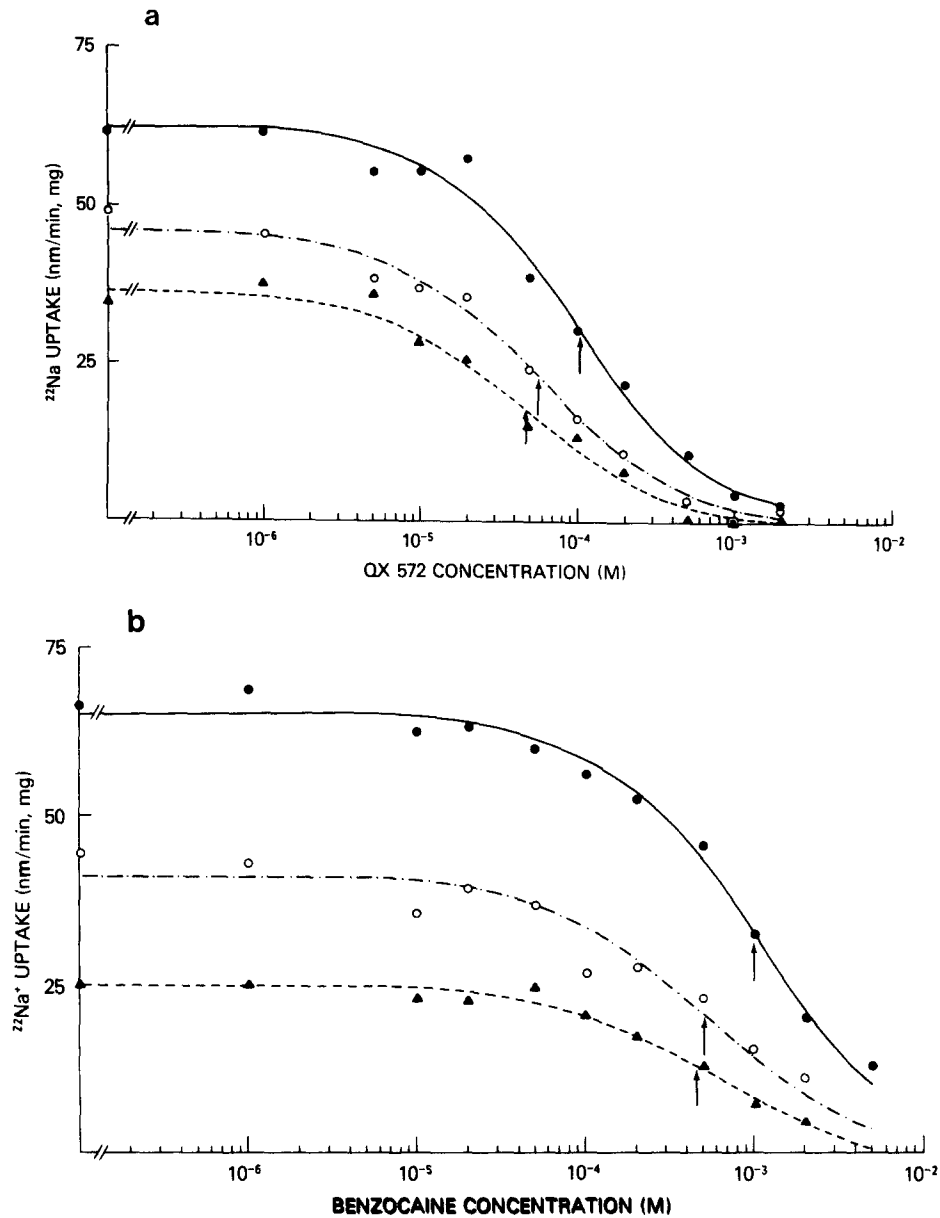


FIGURE 3. (a) Effect of benzocaine on QX 572 inhibition of BTX-activated Na^+ uptake. Cells were incubated in preincubation media containing $1 \mu\text{M}$ BTX, the indicated concentration of QX 572, and 0 (●), 3×10^{-4} M (○), and 7×10^{-4} M (▲) benzocaine for 60 min. Na^+ uptake was measured in the assay medium for 30 s. The solid line is the least squares fit to the modified Michaelis-Menten equation. The arrows indicate the K_i of QX 572 for each benzocaine concentration. (b) Effect of QX 572 on benzocaine inhibition of BTX-activated Na^+ uptake. The experimental procedure is the same as in a. QX 572 concentrations were 0 (●), 6.25×10^{-5} M (○), and 1.46×10^{-4} M (▲).

local anesthetic is added. This agrees with the observation of Mrose and Ritchie (1978) that a mixture of charged and uncharged local anesthetics is more effective in reducing the amplitude of the action potential than is a single local anesthetic.

Next, we would like to consider possible models that would account for the increased effectiveness of mixtures of local anesthetics (Fig. 3). The specific models considered are the following:

(a) One-Site Model

The binding of the charged local anesthetic, the uncharged local anesthetic, and BTX are all mutually exclusive. In the simplest case, there is only one binding site for all these molecules, but there may be more than one binding site if there are allosteric interactions. The only requirement of this model is the mutual exclusivity of binding of the three molecules.

(b) Two-Separate-Site Model

Each channel has two binding sites for local anesthetics: one site for charged local anesthetics and a separate site for uncharged local anesthetics. The two local anesthetics can simultaneously bind to the same channel. The binding of either of these drugs to its site and the binding of BTX are mutually exclusive.

(c) Intermediate Model

Each channel has two binding sites for local anesthetics: one site (site A) for either charged or uncharged local anesthetics, and the other site (site B) for uncharged local anesthetics only. Thus, each channel has one site for charged local anesthetics but two sites for uncharged local anesthetics. The affinities of the two sites for the uncharged drug are assumed to be equal. Charged and uncharged local anesthetics compete for site A. The occupancy of site A and/or site B prevents the binding of BTX. This model is very similar to that proposed by Mrose and Ritchie (1978).

If we generalize model *c* by allowing the uncharged local anesthetic to have different affinities for its two sites, then models *a*, *b*, and *c* can all be considered special cases of this generalized model. The one-site model corresponds to the case in which the affinity of site B for the uncharged local anesthetic is very low. The two-separate-site model corresponds to the case in which the affinity of site A for the uncharged local anesthetic is very low. The intermediate model corresponds to the case in which the affinities of site A and site B for the uncharged local anesthetic are equal. In all cases, we assume that site A and site B are independent.

Explicit dose-response relations for the three models with and without addition of a second drug are derived in the Appendix. For the one-site and two-separate-site models, the apparent dissociation constants are tabulated in Table I. Both of these models give the same apparent dissociation constant in one-drug experiments but different apparent dissociation constants in two-drug experiments. Predictions of the intermediate model depend on which drug is being varied and will be discussed later. Interestingly, each of the three

models gives a very different prediction concerning the change in dose-response curve caused by the addition of a second drug.

As shown in Table I, the one-site model predicts that the addition of a fixed concentration of one local anesthetic would increase the apparent dissociation constant for the dose-response curve of the other local anesthetic. This corresponds to a shift of the dose-response curve to the right. By contrast, the two-separate-site model predicts that the addition of one local anesthetic would decrease the apparent dissociation constant of the other local anesthetic. This corresponds to a shift of the dose-response curve to the left.

Before we describe the predictions of the intermediate model, let us consider the underlying reasons for the qualitative difference between the predictions of the one-site model and the predictions of the two-separate-site model. Let Q represent the drug whose concentration is varied and B the drug that is present at a fixed concentration. For the one-site model, at low dose of Q, the drug B effects a significant reduction in the number of open channels. As the concentration of Q increases, the effectiveness of drug B decreases, until at a very high concentration of Q the presence of drug B has virtually no effect.

TABLE I
THEORETICAL APPARENT DISSOCIATION CONSTANTS

	K_i
1 Drug	$K_Q \left(1 + \frac{[BTX]}{K_{BTX}} \right)$
2-Drug, 1-Site	$K_Q \left(1 + \frac{[BTX]}{K_{BTX}} + \frac{[B]}{K_B} \right)$
2-Drug, 2-Separate-Site	$K_Q \left(\frac{1 + \frac{[BTX]}{K_{BTX}} + \frac{[B]}{K_B}}{1 + \frac{[B]}{K_B}} \right)$

Thus, for the one-site model, drug B lowers the dose-response curve considerably at the left end and very little at the right end. As a result, the normalized dose-response curve shifts to the right, and K_i increases. As shown quantitatively in the Appendix and Table I, the addition of drug B increases K_i by an amount

$$K_Q \left(\frac{[B]}{K_B} \right)$$

(where K_Q and K_B are the true dissociation constants for drug Q and drug B).

In the case of the two-separate-site model, there are two effects that must be considered. First, the presence of a fixed concentration of drug B tends to increase K_i in the two-separate-site model as it does in the one-site model. However, there is an additional effect in the two-separate-site model that tends to decrease K_i . This additional effect results from the availability of two noncompetitive sites, allowing two drug molecules to bind at the same channel simultaneously. The second binding does not affect the flux through the

channel, because the channel is assumed to be completely closed by the binding of the first drug. However, the second binding decreases the probability that the closed channel will open, because opening requires the dissociation of both drug molecules from their sites. This drives the equilibrium toward the closed state. The importance of this additional effect increases as the concentration of the variable drug Q increases, because that increases the number of channels with two drugs bound. Thus, the additional effect lowers the dose-response curve considerably at the right end and very little at the left end. As a result, in experiments with two drugs, the dose-response curve for the two-separate-site model is shifted to the left relative to the dose-response curve for the one-site model. In fact, the additional effect that occurs only in the two-separate-site model is the larger effect. Therefore, in the two-separate-site model, the addition of drug B results in a decrease in K_i . As shown quantitatively in the Appendix and Table I, the amount of this decrease is

$$K_Q \left(\frac{[B][BTX]}{K_B K_{BTX}} \right) / \left(1 + \frac{[B]}{K_B} \right)$$

(where K_{BTX} is the true dissociation constant for BTX).

Next, let us consider the predictions of the intermediate model. For the two-drug experiments, as shown in the Appendix, the dose-response curve has a different form, depending on whether the concentration of the charged or the uncharged local anesthetic is being varied. The dose-response curve has a Michaelis-Menten form when the charged local anesthetic is varied, but does not have this form when the uncharged local anesthetic is varied. For the former case, the apparent dissociation constant is shown in the Appendix (Eq. 6A). For the latter case, the apparent dissociation constant was determined by numerically calculating the normalized dose-response curve, and then evaluating the dose where the normalized response equals 0.5. In each case, the predictions of the intermediate model for one-drug experiments were determined by setting the fixed-drug concentration equal to zero.

A convenient parameter for comparing the predictions of the three models with the experiments of Fig. 3 is the ratio of the apparent dissociation constant for a one-drug experiment to the apparent dissociation constant after a fixed concentration of the other drug is added. This ratio is a measure of the shift of the dose-response curve when the second drug is added. Ratios less than 1 correspond to shifts to the right, and ratios greater than 1 correspond to shifts to the left.

Table II compares experimental values of the apparent dissociation constant ratio with the predictions of the three models for four different experimental conditions. For each condition, the experimental ratios are near 2, corresponding to substantial shifts of the dose-response curves to the left. The one-site model and the intermediate model predict ratios less than or about equal to 1, corresponding to shifts of the dose-response curve to the right, or essentially no shift. Only the two-separate-site model predicts a ratio near 2, corresponding to substantial shifts of the dose-response curve to the left, and in good agreement with experiment. This demonstrates unambiguously that QX 572 and benzocaine act at separate sites in the sodium channel.

Where are these binding sites for the local anesthetics located? Two different sites of action have been proposed to account for the reduction of sodium conductance by local anesthetics. First, the local anesthetics may bind directly to a specific site on the gating molecules. The binding holds the sodium gates in the closed conformation or inactive state and thus decreases Na^+ permeability (Huang et al., 1978; Lipicky et al., 1978). The alternative hypothesis is that the local anesthetics may bind in the lumen of the Na^+ channel. The binding would decrease Na^+ permeability by occluding the channel (Strichartz, 1973; Courtney, 1975; Hille, 1977 *b*; Cahalan and Almers, 1979). The results reported in this paper are consistent with either of these hypotheses, and do not distinguish between them. In either case, the receptor sites for the local anesthetics must be coupled with gating molecules in such a way that the binding of these drugs profoundly affect both sodium inactivation and BTX binding.

We have only tested two specific local anesthetics so far. Whether our results apply to charged and uncharged local anesthetics in general is not yet known, but the consistency of our results with the findings of Mrose and Ritchie

TABLE II
COMPARISON OF EXPERIMENTAL AND PREDICTED APPARENT
DISSOCIATION CONSTANT RATIOS*

	Dose-response curve for benzocaine		Dose-response curve for QX 572	
	6.25×10^{-5} M QX	1.46×10^{-4} M QX	3.0×10^{-4} M Benz	7.0×10^{-4} M Benz
Experimental	2.0	2.2	1.8	2.1
One-site model	0.6	0.4	0.8	0.6
Intermediate model	1.0	1.1	1.0	1.0
Two-site model	1.9	2.4	1.6	2.1

* Ratio of K_i for one-drug experiment to K_i in presence of second drug.

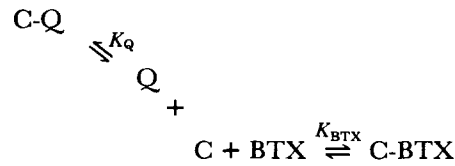
(1978) in their work with lidocaine, marcaine, and benzocaine suggest the possible generality of the two-separate-site model.

The finding that there are two separate sites for local anesthetics in the sodium channel suggests the possible clinical usefulness of obtaining a given degree of channel blocking by using mixtures of two drugs—each drug binding to a different receptor. As previously indicated, the binding of two different drugs at two separate sites in a channel decreases the probability that a closed channel will open, thus driving the equilibrium toward the closed state. Thus, the use of two drugs might reduce the required dose, particularly if a large fraction of channels needs to be blocked. As a result, there may be a reduction in local anesthetic side effects.

APPENDIX

One Drug

For the case in which one local anesthetic competes with BTX for a single class of binding sites, the equilibrium can be described as follows:



where C represents unbound channels, C-BTX represents channels with BTX bound and C-Q represents channels with local anesthetic Q bound. The dissociation constants are related to the concentrations of the drugs as follows:

$$K_{\text{BTX}} = \frac{[\text{C}][\text{BTX}]}{[\text{C-BTX}]}$$

$$K_Q = \frac{[\text{C}][\text{Q}]}{[\text{C-Q}]}$$

The total concentration of channels is $[\text{C}_T] = [\text{C}] + [\text{C-BTX}] + [\text{C-Q}]$. Since the measured flux is proportional to the concentration of channels with BTX bound,

$$\text{Flux} \propto [\text{C-BTX}] = \frac{[\text{C}][\text{BTX}]}{K_{\text{BTX}}}$$

Substituting the value of [C] from the preceding equations,

$$\text{Flux} \propto \frac{[\text{BTX}][\text{C}_T]}{K_{\text{BTX}} \left(1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{Q}]}{K_Q} \right)}$$

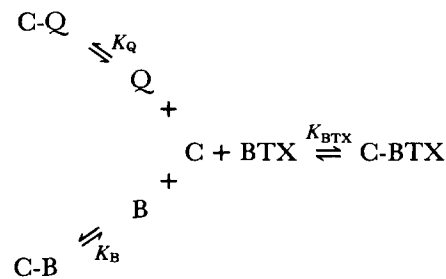
For a given value of [BTX] and a given preparation, the maximum flux corresponds to $[\text{Q}] = 0$. Therefore,

$$\frac{\text{Flux}}{\text{Max flux}} = \frac{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}}}{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{Q}]}{K_Q}} \tag{1A}$$

Eq. 1A is the normalized dose-response curve for one local anesthetic drug (Q) competing with BTX for a single class of sites.

Two Drugs, One-Site Model

For the case where two local anesthetics compete with BTX for a single class of binding sites, the equilibrium can be described as follows:



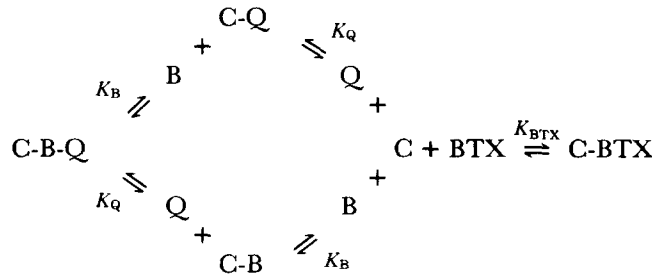
where C-B represents channels with local anesthetic B bound and the other symbols

are the same as described. Using reasoning similar to that employed for the one-drug model, the normalized dose-response curve for two local anesthetic drugs competing with BTX for a single class of sites is:

$$\frac{\text{Flux}}{\text{Max flux}} = \frac{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}}}{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{Q}]}{K_{\text{Q}}}} \quad (2A)$$

Two Drugs, Two-Separate-Site Model

Next, we consider the case in which there are two sites at each channel; one binds only charged local anesthetics and the other binds only uncharged local anesthetics. Binding at either of these sites and binding of BTX are mutually exclusive. The equilibrium for this case can be described as follows:

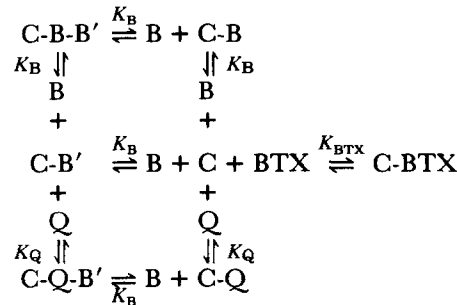


where C-B-Q represents channels with both local anesthetics bound and the other symbols are the same as described. For simplicity, we have assumed that the two sites are independent. Therefore, the normalized dose-response curve for this case is

$$\frac{\text{Flux}}{\text{Max flux}} = \frac{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}}}{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{Q}]}{K_{\text{Q}}} \left(1 + \frac{[\text{B}]}{K_{\text{B}}} \right)} \quad (3A)$$

Two Drugs, Intermediate Model

In this case, there are two sites for each channel: one binds either charged local anesthetics or uncharged local anesthetics, and the other binds only uncharged local anesthetics. Binding at either of these sites and binding of BTX are mutually exclusive. The equilibrium for this case can be described as follows:



where C-B-B' represents channels with uncharged local anesthetic bound at both sites. The normalized dose-response curve for Q when a fixed amount of B is added is

$$\frac{\text{Flux}}{\text{Max flux}} = \frac{\left(1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]^2}{K_{\text{B}}K_{\text{B}}}\right)}{\left(1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]^2}{K_{\text{B}}K_{\text{B}}}\right) + \frac{[\text{Q}]}{K_{\text{Q}}}\left(1 + \frac{[\text{B}]}{K_{\text{B}}}\right)} \quad (4A)$$

The normalized dose-response curve for B with the addition of the fixed amount of Q is

$$\frac{\text{Flux}}{\text{Max flux}} = \frac{1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{Q}]}{K_{\text{Q}}}}{\left(1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]^2}{K_{\text{B}}K_{\text{B}}}\right) + \frac{[\text{Q}]}{K_{\text{Q}}}\left(1 + \frac{[\text{B}]}{K_{\text{B}}}\right)} \quad (5A)$$

Apparent Dissociation Constants

Eqs. 1A, 2A, 3A, 4A, and 5A describe the decrease in flux as the concentration of a local anesthetic is increased. Since Eqs. 1A, 2A, 3A, and 4A are of the modified Michaelis-Menten form, the apparent dissociation constants can be simply determined by writing these equations in the standard form:

$$\text{Relative flux} = \frac{K_i}{K_i + [\text{Q}]}$$

The apparent dissociation constants for Eqs. 1A, 2A, and 3A are tabulated in Table I. The apparent dissociation constants for the intermediate case with the uncharged drug concentration fixed and the charged drug concentration varied is:

$$K_i = \frac{K_{\text{Q}}\left(1 + \frac{[\text{BTX}]}{K_{\text{BTX}}} + \frac{2[\text{B}]}{K_{\text{B}}} + \frac{[\text{B}]^2}{K_{\text{B}}^2}\right)}{1 + \frac{[\text{B}]}{K_{\text{B}}}} \quad (6A)$$

For the intermediate case with the uncharged drug concentration varied, the equation (Eq. 5A) is not of the Michaelis-Menten form.

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