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Inhibition of Binding of [^{3}H]Batrachotoxinin A 20-α-Benzoate to Sodium Channels by Local Anesthetics

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SUMMARY

The effects of several local anesthetics on the binding of ligands to receptors associated with voltage-sensitive sodium channels in rat brain synaptosomes have been examined. In the presence of 0.3 μM scorpion toxin, the 13 local anesthetics tested inhibited the specific binding of [^{3}H]batrachotoxinin A 20α-benzoate ([^{3}H]BTX-B), a ligand which binds to a receptor site responsible for the activation of sodium channel ion flux, in a dose-dependent fashion, with K_D values ranging from 1.2 μM for tetracaine to 1.58 mM for benzocaine. A plot of log K_D from these binding experiments against log $K_{0.5}$ for inhibition of sodium currents by local anesthetics from electrophysiological experiments yielded a regression line with a slope of 0.84 and a correlation coefficient, r, of 0.86, demonstrating that the inhibition of [^{3}H]BTX-B binding by local anesthetics occurs within a concentration range of physiological relevance. Tetracaine had little effect on basal ^{125}I-labeled scorpion toxin binding to synaptosomes in the absence of batrachotoxin. However, in the presence of batrachotoxin, tetracaine inhibited the batrachotoxin-dependent increase in scorpion toxin binding ($K_D = 2.0$ μM) in a dose-dependent manner, suggesting that inhibition of [^{3}H]BTX-B binding by local anesthetics does not occur through binding at the scorpion toxin binding site. The inhibition of [^{3}H]BTX-B binding by lidocaine was reversible within 30 min when samples were diluted from 10^{-3} M to 10^{-4} M lidocaine. Scatchard analysis of [^{3}H]BTX-B binding to synaptosomes showed that bupivacaine and tetracaine reduced receptor affinity without decreasing maximal binding capacity. This reduction in receptor affinity in the presence of local anesthetics appears to be due, at least in part, to an increased rate of ligand dissociation from the receptor-ligand complex, suggesting an indirect allosteric mechanism for the inhibition of [^{3}H]BTX-B binding by local anesthetics. Analysis of the effects of local anesthetics in terms of an allosteric model of drug action showed that they bind to inactive states of sodium channels with at least a 10-fold higher affinity than active states. A 7-fold difference in K_D for inhibition of [^{3}H]BTX-B binding between the local anesthetic stereoisomers RAC 109 I and RAC 109 II was observed. Similarly, the dissociation rate constant for the [^{3}H]BTX-B/receptor complex was increased 9.3-fold in the presence of RAC 109 II and 4.3-fold in the presence of a comparable concentration of RAC 109 I. Taken together, the results suggest that the action of local anesthetics involves interaction with a specific receptor site(s) involved in the activation of voltage-sensitive sodium channels. Since this interaction is stereospecific, this putative receptor site(s) probably includes protein components of the sodium channel. Occupancy of the local anesthetic receptor site blocks batrachotoxin binding by an indirect allosteric mechanism.

INTRODUCTION

Because of extensive electrophysiological investigations, much is known regarding the effects of local anesthetics on sodium channels in nerve membranes (1). Application of the voltage clamp technique has provided a detailed description of local anesthetic action in squid giant axon (2). Moreover, correlations of local anesthetic structure and activity have been made (3). The ability of local anesthetics to inhibit ionic flow through sodium channels correlates well with their pharmacological activity, indicating that the sodium channel is the site of local anesthetic action. However, because the sodium

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channel is located in the cell membrane, it is uncertain whether local anesthetic action involves interaction with a specific receptor site on the sodium channel protein or a primary effect on the lipid phase of the cell membrane that alters ionic channel properties indirectly (4).

Development of radiolabeled neurotoxins which serve as molecular probes interacting specifically with the sodium channel (for review see ref. 5) has provided a new approach to study local anesthetic action at the molecular level. Voltage-sensitive sodium channels have at least three separate receptor sites for neurotoxins associated with protein components of the channels. The inhibitors tetrodotoxin and saxitoxin bind at receptor site 1 and inhibit ion flux through the sodium channel (6). The alkaloids veratridine, BTX,[3] and aconitine act at receptor site 2 to cause persistent activation of sodium channels (5). The polypeptides scorpion toxin and sea anemone toxin bind at receptor site 3 (5). These toxins slow sodium channel inactivation (reviewed in ref. 5), enhance persistent activation of sodium channels by toxins acting at site 2 through an allosteric mechanism (5), and enhance binding of ligands at site 2 (7, 8).

Application of these toxins has yielded additional information regarding local anesthetic action. Local anesthetics do not affect saxitoxin or tetrodotoxin binding at neurotoxin receptor site 1 (9, 10) or scorpion toxin binding at neurotoxin receptor site 3 (10) at concentrations that effectively block sodium channels. In contrast, early electrophysiological studies showed that procaine could block the irreversible depolarization of nerve and muscle by BTX, suggesting that procaine prevents BTX binding (11). In frog nerve, several local anesthetics inhibit BTX-activated sodium currents (12, 13). Moreover, procaine blocks channels unmodified by BTX at lower concentration than BTX-activated sodium channels, suggesting a competitive interaction between local anesthetics and BTX (12). In a study of ^{22}Na uptake in neuroblastoma cells, lidocaine inhibited BTX-activated sodium influx in a manner consistent with a competitive allosteric mechanism (10). Similar studies in neuroblastoma cells with the local anesthetics benzocaine and QX 572 have also shown that local anesthetics interact competitively with BTX (14).

Recent synthesis of a tritiated derivative of BTX, [^{3}H] BTX-B (15), development of convenient methods for measuring specific binding of [^{3}H]BTX-B to intact synaptosomes (8), and successful application of this radioligand in a study of anticonvulsant drug effects on sodium channels (16) prompted us to examine the effects of local anesthetics on [^{3}H]BTX-B binding directly. While our experiments on sodium channels in synaptic nerve endings were in progress, Creveling *et al.* (17) reported related results on a microsac preparation consisting principally of neuronal cell ghosts from a rodent brain.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained as follows: tetrodotoxin from Calbiochem (San Diego, Calif.); veratridine from Aldrich Chemical Company (Milwaukee, Wisc.); scorpion (*Leiurus quinquestriatus*) venom, bovine serum albumin, and aconitine from Sigma Chemical Company (St. Louis, Mo.). BTX was a gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, National Institutes of Health), and [^{3}H]BTX-B was graciously supplied by Dr. George Brown (University of Alabama, Birmingham, Ala.). The preparation and purification of BTX and [^{3}H]BTX-B has been described elsewhere (15). Scorpion toxin was purified and labeled with ^{125}I, using a lactoperoxidase-catalyzed iodination as previously described (18, 19). All local anesthetic compounds were obtained with the generous cooperation of Dr. Bertil Takman, of Astra Pharmaceuticals (Worcester, Mass.). Solutions of benzocaine were prepared by dissolving the compound in ethanol followed by dilution into aqueous medium with gentle warming. Control experiments showed that the final concentration of ethanol (0.372%) had little effect on [^{3}H]BTX-B binding. The other local anesthetics (received as the hydrochloride salts) were readily soluble in water. Fresh stock solutions were made daily and diluted in assay medium as required. All other standard laboratory reagents were of analytical grade.

Preparation of Synaptosomes. Synaptosomes were prepared from whole rat brain according to a modification of the method of Gray and Whittaker (20). The brains of male Sprague-Dawley rats were removed and homogenized in ice-cold 0.32 M sucrose using 10 complete excursions in a motor-driven Teflon-glass homogenizer. All subsequent procedures were performed at 4°. The homogenate (10% w/v) was centrifuged at 1,000 × *g* for 10 min and the resultant supernatant (S_1) was retained. The pellet (P_1) consisting mainly of sedimented nuclei, blood cells, and broken cellular fragments, was resuspended (10% v/v) in 0.32 M sucrose and recentrifuged as above. The supernatant (S_2) was combined with S_1 and centrifuged at 17,000 × *g* for 60 min. The supernatant was discarded, and the pellet (P_3) was resuspended in 0.32 M sucrose containing 5 mM KH_2O_4 (pH 7.4) to a volume of 10 ml. The resuspended P_3 fraction was then layered onto a discontinuous gradient consisting of 10-ml layers of 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose containing 5 mM K_2HPO_4 (pH 7.4). The gradient was centrifuged at 100,000 × *g* (maximal radius, SW 25.2 rotor) for 105 min. The synaptosome fraction sedimenting at the 1.0–1.2 M sucrose interface was collected and diluted to 0.32 M sucrose by the dropwise addition of 5 mM K_2HPO_4 (pH 7.4) during constant stirring. The diluted synaptosomal fraction was centrifuged at 40,000 × *g* for 45 min, and the resultant pellet was resuspended in standard incubation medium (consisting of 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes-Tris (pH 7.4), and 130 mM choline chloride) to a final protein concentration ranging from 15 to 25 mg/ml using a loose-fitting glass-glass homogenizer. The synaptosomes were divided into 0.5- to 1.0-ml aliquots and slowly frozen over 30 min on dry ice prior to storage in liquid nitrogen. Previous studies have shown that there is no appreciable loss of toxin binding activity or $^{22}Na^+$ flux activity using this procedure (21).

Measurement of radiolabeled neurotoxin binding. Specific binding of ^{125}I-scorpion toxin and [^{3}H]BTX-B was measured as described previously (8, 22). Prior to use, an aliquot of frozen synaptosomes was warmed in a 36° water bath until thawed (3–4 min) and then stored on ice. The synaptosomes were diluted 4-fold in standard incubation medium (5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes-Tris (pH 7.4), and 130 mM choline chloride) to a final protein concentration of 1.25 mg/ml in the assay. ^{125}I-Scorpion toxin binding was measured by incubating synaptosomes for 10 min at 36° in the standard incubation medium containing 25 mM KCl and 105 mM choline chloride plus ^{125}I-scorpion toxin (0.1 nM), tetrodotoxin (1 μM), BSA (1 mg/ml), in the absence or presence of batrachotoxin (1.25 μM), and varying concentrations of added drug. Nonspecific binding of ^{125}I-scorpion toxin was determined in the presence of 200 nM unlabeled scorpion toxin as described previously (22). Binding reactions were initiated by the addition of 25 μl of the synaptosomal suspension to 175 μl of the reaction mixture. Samples were rapidly mixed and, following incubation for 10 min, the reaction was stopped by the addition of 3 ml of ice-cold wash medium consisting of 163 mM choline chloride, 5 mM Hepes-Tris

[3] The abbreviations used are: BTX, batrachotoxin; BTX-B, batrachotoxinin-A 20-α-benzoate; Hepes, 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid; BSA, bovine serum albumin.

(pH 7.4), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and BSA (1 mg/ml). The synaptosomes were immediately collected on glass-fiber filters (Whatman GF/C) under vacuum and washed three times with 3 ml of wash medium. ^{125}I bound to filters was determined in a gamma scintillation spectrometer.

[^{3}H]BTX-B binding was studied as described previously (8). Synaptosomes were incubated for 30 min at 36° in the standard incubation medium plus [^{3}H]BTX-B (10 nM), tetrodotoxin (1 μM), scorpion toxin (0.3 μM), BSA (1 mg/ml), and varying concentrations of added drugs. Binding reactions were started by the addition of 50 μl of synaptosomal suspension to 150 μl of the reaction mixture. Synaptosomes were collected and washed as above. Filters containing trapped synaptosomes were suspended in liquid scintillation fluid, and ^{3}H bound was determined in a liquid scintillation counter. Nonspecific binding of [^{3}H]BTX-B was determined in the presence of 0.3 mM veratridine or 0.3 mM aconitine. As in previous experiments using this technique (8), nonspecific binding comprised 15–20% of total binding at 10 nM [^{3}H]BTX-B (see fig. 1 of ref. 8). Nonspecific binding was unaffected by the presence of local anesthetics at the highest concentrations studied. However, the estimates of nonspecific binding varied slightly depending upon whether veratridine or aconitine was used to define it. On average, nonspecific binding determined in the presence of veratridine was 10% smaller than that in the presence of aconitine. Generally these two values were averaged to give a best estimate of nonspecific binding.

Other methods. Synaptosomal protein concentration was measured according to the method of Peterson (23). Linear segments on Scatchard plots, Hill plots, and kinetic experiments were computed by linear regression. Using an iterative least-squares method, selected data were fit with a computer to equations describing an allosteric model for local anesthetic action on the sodium channel.

RESULTS

Local anesthetic inhibition of [^{3}H]BTX-B binding to synaptosomes. The effect of three drugs with local anesthetic properties [tetracaine (●), propranolol (○), and lidocaine (△)] on specific binding of [^{3}H]BTX-B is illustrated in Fig. 1. The drugs have no effect on nonspecific binding. Local anesthetic inhibition of specific toxin binding is dose-dependent, with $K_{0.5}$ values of 1.2 μM, 11.0 μM, and 300 μM for tetracaine, propranolol, and lidocaine, respectively. Since the concentration of [^{3}H]BTX-B (10 nM) is substantially below its K_D, these values of $K_{0.5}$ are essentially equal to K_D. The results illustrated in Fig. 1 are typical of those for 13 local anesthetics that were studied. Mean values for K_D and Hill coefficient for block of [^{3}H]BTX-B binding by these drugs are listed in Table 1. A range of K_D values from 1.7 μM to 1.78 mM was observed.

TABLE 1

Effect of local anesthetics on BTX-B binding

Local anesthetic	Hill coefficient	K_D (BTX-B binding)	$K_{0.5}$ (electrophysiological data)[a]
		μM	μM
Tetracaine	0.99	1.7 ± 0.6	0.31
Etidocaine	0.63	3.6 ± 1.2	7.1
Bupivacaine	0.94	4.5 ± 1.8	31
(±)-Propranolol	1.06	15.5 ± 4.5	28
(+)-Propranolol			
Prilocaine	0.80	50.0 ± 3.1	556
Mepivacaine	0.88	192 ± 19	140
Procaine	0.97	161 ± 73	90
Tocainide	0.85	241 ± 41	636
Lidocaine	0.93	311 ± 53	140
W 36017	0.86	1100 ± 100	426
Benzocaine	1.61	1785 ± 86	2300

[a] $K_{0.5}$ was calculated by applying equation 3 of ref. 4 to data taken from ref. 4 and, for benzocaine, from ref. 24.

In order to determine whether the inhibition of sodium channel ion conductance by local anesthetics results from the same mechanism of action as block of [^{3}H]BTX-B binding, we have compared (Table 1) the K_D values derived from our neurotoxin binding measurements with those derived from electrophysiological experiments under conditions of tonic block (3). These data indicate that there is a significant correlation of the two parameters over a 1000-fold range of $K_{0.5}$ values. This conclusion is confirmed by a plot of log K_D for drug binding against log $K_{0.5}$ for channel blockade (Fig. 2).

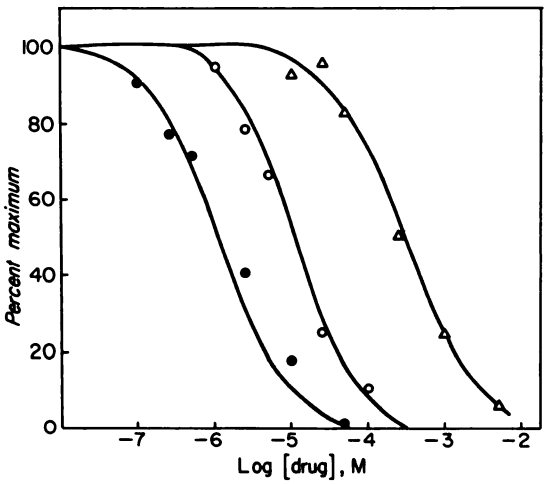


FIG. 1. *Effect of local anesthetics on the binding of [^{3}H]BTX-B to synaptosomes*

Synaptosomes were incubated in the presence of 10 nM [^{3}H]BTX-B (in the presence of 0.3 μM scorpion toxin) and varying concentrations of tetracaine (●), propranolol (○), and lidocaine (△) for 30 min. Binding was then measured as described under Experimental Procedures. The results of a single experiment performed with triplicate samples are presented. They are representative of two or three experiments for each drug.

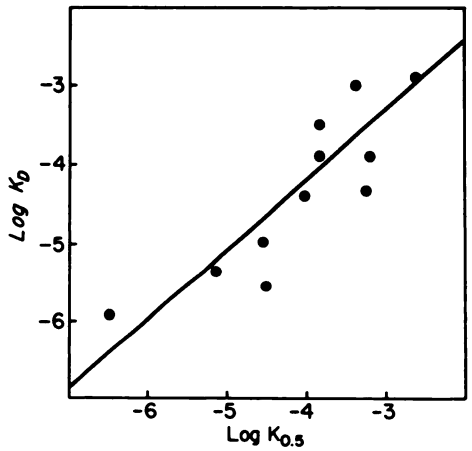


FIG. 2. *Correlation of K_D from inhibition of BTX-B binding with $K_{0.5}$ from electrophysiological data*

Data taken from binding inhibition curves are plotted as log K_D for inhibition of BTX-B binding against log $K_{0.5}$ for inhibition of sodium currents in electrophysiological experiments.

The regression line has a slope of 0.84 and a correlation coefficient, r, of 0.86.

Estimation of the affinity for local anesthetic binding and action is complicated by the frequency dependence of these parameters (25). Local anesthetic inhibition of sodium channels is more rapid and complete if nerve preparations are repetitively depolarized to cause channel activation (26, 27). These effects are thought to result from rapid binding of the drugs to the ion-conducting state(s) of sodium channels together with high-affinity binding to the inactivated state(s) (25). Since sodium channels cannot be repetitively activated during our binding assays, we have compared our binding data with values for tonic inhibition, the extent of channel block at very low frequencies of stimulation. Since a good correlation is observed (Fig. 2), it is likely that tonic block of sodium channels by local anesthetics also prevents [^{3}H]BTX-B binding. Creveling *et al.* (17) observed a close correlation between block of BTX-induced depolarization of brain microsacs and block of [^{3}H]BTX-B binding by local anesthetics, providing additional support for the conclusion that these two effects result from the same mechanism of action.

As can be seen from Table 1, Hill coefficients for inhibition of [^{3}H]BTX-B binding by most of the local anesthetics are about 1.0, suggesting that local anesthetics interact with a single class of binding sites. There are two notable exceptions: etidocaine and benzocaine. The Hill coefficient for etidocaine (0.63) suggests multiple sites of drug action with differing affinities or negatively cooperative interactions. The benzocaine data yield a Hill coefficient which suggests positive cooperativity ($n_H = 1.61$). Further work is required to determine whether this difference in Hill coefficient reflects fundamental differences in mechanism of action or experimental uncertainty in controlling the free concentration of these highly lipid-soluble anesthetics.

Effect of local anesthetics on binding of scorpion toxin to synaptosomes. In view of the allosteric coupling which has been observed between neurotoxin receptor sites 2 and 3 on the sodium channel (5, 8), inhibition of [^{3}H]BTX-B binding by local anesthetics may have resulted from a primary effect on the scorpion toxin site. Consequently, local anesthetic effects at the site 3 were examined. Binding of scorpion toxin to neurotoxin receptor site 3 can be measured directly by using ^{125}I-scorpion toxin (22). In synaptosomes, scorpion toxin binding is enhanced by BTX (8). The enhancement is greater in depolarized synaptosomes. Thus, in medium containing 25 mM K^+ to partially depolarize synaptosomes, the effect of local anesthetics on scorpion toxin binding can be measured directly and compared with the effect of these drugs on the BTX enhancement of scorpion toxin binding. The results of Fig. 3 show that tetracaine has little effect on specific scorpion toxin binding. In the presence of 1.25 μM batrachotoxin, scorpion toxin binding is enhanced approximately 50%, similar to data previously reported (16). Tetracaine produces a concentration-dependent inhibition of that portion of ^{125}I-labeled scorpion toxin binding which is enhanced by the alkaloid toxin with a mean IC_{50} of 4.7 μM, corresponding to a K_D value of 1.7 ± 0.3 μM. This value compares favorably with the K_D of tetracaine for inhibition of [^{3}H]BTX-B to synaptosomes of 1.3 μM. These results show that local anesthetics do not decrease BTX binding through a primary effect on site 3 but can inhibit scorpion binding enhanced by batrachotoxin.

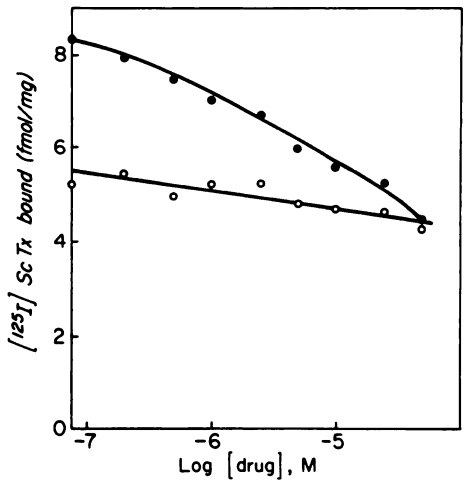


FIG. 3. *Effect of tetracaine on ^{125}I-scorpion toxin ([^{125}I] Sc Tx) binding*

Synaptosomes were incubated for 10 min in medium containing 0.1 nM ^{125}I-labeled toxin [in the absence (○) or presence (●) of 1.25 μM unlabeled BTX] and varying concentrations of tetracaine. The concentration of KCl in the medium was 25 mM. Binding was then measured as described under Experimental Procedures. The results presented represent data of a single experiment in which triplicate determinations were made. The IC_{50} was 5.6 μM, corresponding to a K_D of 2.0 μM. The mean values (± standard error of the mean) of three comparable experiments were IC_{50} = 4.7 μM ± 0.9 μM and K_D = 1.7 μM ± 0.3 μM.

The results described thus far suggest that the effects of local anesthetics on the sodium channel occur through, or at least have effects on, the BTX receptor site. The nature of this interaction is explored further in the following sections.

Reversibility of local anesthetic inhibition of [^{3}H]BTX-B binding. In order to examine the reversibility of inhibition of [^{3}H]BTX-B binding by local anesthetics, synaptosomes were incubated for 30 min in the presence of 10^{-3} M lidocaine, diluted 10-fold to 10^{-4} M lidocaine while simultaneously adding 10 nM [^{3}H]BTX-B, and assayed after 30 and 45 min to observe the association of [^{3}H]BTX-B to its receptor. Table 2 illustrates the results of this study along with control measurements in which synaptosomes had been incubated in the presence of 10^{-3} or 10^{-4} M lidocaine throughout. As expected from the inhibition curve for lidocaine (Fig. 1), little binding of [^{3}H]BTX-B is observed at 10^{-3} M lidocaine. In the presence of 10^{-4} M lidocaine, [^{3}H]BTX-B binding increases to 49 fmoles/mg, approaching equilibrium in approximately 30–45 min as in previous studies (8, 16). In the samples which were diluted from 10^{-3} M to 10^{-4} M lidocaine, [^{3}H]BTX-B binding reached values comparable to the samples in 10^{-4} M lidocaine. This shows that the reduction of [^{3}H]BTX-B binding by lidocaine is fully reversible within 30 min and therefore that lidocaine does not irreversibly alter the affinity or number of BTX receptors.

TABLE 2

Reversibility of lidocaine inhibition

Synaptosomes were incubated for 30 min in the presence of 0.3 μM scorpion toxin and 10^{-3} M or 10^{-4} M lidocaine as indicated. At the end of Incubation 1, 10 nM [^{3}H]BTX-B was added and Incubation 2 was continued for 30 or 45 min in the presence of 10^{-3} M or 10^{-4} M lidocaine as indicated. Bound [^{3}H]BTX-B was then measured as described under Experimental Procedures. The results represent the mean ± standard deviation of three experiments.

Lidocaine concentration		[^{3}H]BTX-B bound		n
Incubation 1	Incubation 2	30 min	45 min	
M		*fmoles/mg*		
1×10^{-4}	1×10^{-4}	41.7 ± 4.5	49.1 ± 7.3	3
1×10^{-3}	1×10^{-3}	0	2.9 ± 1.2	3
1×10^{-3}	1×10^{-4}	41.1 ± 12.2	44.7 ± 6.3	3

Effects of local anesthetics on equilibrium and kinetic properties of [^{3}H]BTX-B binding. Scatchard analysis (with 300 μM scorpion toxin) of labeled BTX binding over an extended concentration range (Fig. 4) showed that this ligand binds to a single class of high-affinity sites with a K_D of 116 nM, similar to that reported previously (8). Bupivacaine (6.2 μM) increased the K_D for [^{3}H]BTX-B from 116 nM to 312 nM. The maximal binding capacity in this experiment (1.9 pmoles/mg) is little affected. Similar results were obtained in two additional experiments using 2.6 μM bupivacaine (see legend to Fig. 4) and also for tetracaine (data not illustrated). These results indicate that the inhibition of [^{3}H]BTX-B binding by local anesthetics is competitive, resulting in an increase in K_D without reduction in binding capacity. However, in occasional experiments with higher local anesthetic concentrations, we have observed a moderate (up to 20%) increase in binding capacity in the presence of local anesthetics depending on whether veratridine or aconitine was used to determine nonspecific binding. We have never observed a decrease in binding capacity. This increase probably reflects uncertainty in the precise determination of nonspecific binding using veratridine and aconitine as discussed under Experimental Procedures. This uncertainty becomes important only when specific binding is greatly reduced in the presence of a high concentration of local anesthetic. In any case, the data indicate a competitive mechanism for inhibition of [^{3}H]BTX-B binding by local anesthetics.

Figure 5 illustrates the dissociation of the [^{3}H]BTX-B/receptor complex in the presence of the competitive ligand aconitine or aconitine plus local anesthetics at concentrations about 10 times greater than their K_D values. A range of dissociation rates is observed with local anesthetics. In each case, local anesthetics significantly increase the dissociation rate over that with a saturating concentration of aconitine alone. Dissociation rate constants and half-lives for these dissociation reactions are summarized in Table 3. The increase in dissociation rate constant over that with aconitine ranges from 2.3-fold for tetracaine to 8.5-fold for lidocaine. These results indicate that local anesthetics act as indirect allosteric competitive inhibitors of BTX binding.

Stereospecificity in local anesthetic binding. The experimental local anesthetics RAC 109 I and RAC 109 II are

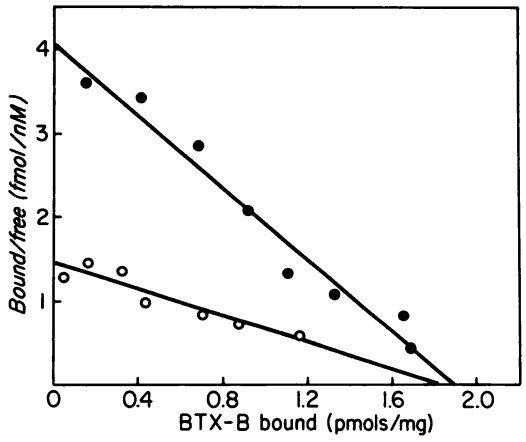


FIG. 4. *Scatchard analysis of [^{3}H]BTX-B binding to synaptosomes*

Specific binding of 10 nM [^{3}H]BTX-B was measured in standard incubation medium (scorpion toxin concentration = 0.3 μM) in the absence (●) or presence (○) of 6.2 μM bupivacaine, with increasing concentrations of unlabeled BTX as described under Experimental Procedures. Total BTX-B specifically bound (labeled plus unlabeled) was calculated and plotted as a Scatchard plot. The results presented represent data from a single experiment in which triplicate determinations were made. Best-fit values of B_{max} were 1.9 pmoles/mg in both the presence and absence of drug while the K_D was increased from 116 nM in the absence of bupivacaine to 312 nM in its presence. In two additional experiments with a different synaptosome preparation, the B_{max} was 1.3 ± 0.17 pmoles/mg in the absence of bupivacaine and 1.38 ± 0.16 pmoles/mg in the presence of 2.6 μM bupivacaine. The K_D was increased from 65 ± 9 nM to 110 ± 10 nM in the presence of 2.6 μM bupivacaine.

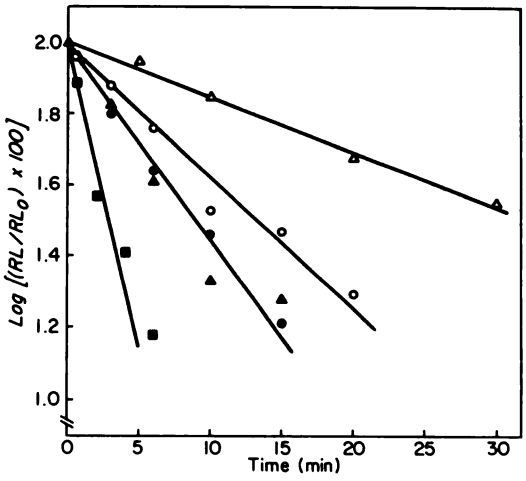


FIG. 5. *Time course of dissociation of the [^{3}H]BTX-B/receptor complex*

Synaptosomes were incubated in standard incubation medium for 30 min with 10 nM [^{3}H]BTX-B and, at zero time, 200 μM aconitine alone (△) or 200 μM aconitine plus 13 μM tetracaine (○), 470 μM prilocaine (●), 1.6 mM tocainide (△), or 2.5 mM lidocaine (▲) was added. Owing to the limited solubility of aconitine, 1% ethanol was present in all samples. At the indicated times, samples were removed and bound [^{3}H]BTX-B was determined as described under Experimental Procedures. The results represent the combined data of four experiments, each performed in duplicate. Two additional series of experiments gave similar increases in dissociation rate constant by local anesthetics.

TABLE 3

Kinetic data for the dissociation of the [^{3}H]BTX-B/receptor complex in the presence of local anesthetics and/or aconitine

Ligand	Concentration	Dissociate rate constant (k_{-1})	Half-life ($t_{1/2}$)
	μM	min^{-1}	min
Aconitine	200	0.0156	19.3
Tetracaine	13	0.0354	8.5
Prilocaine	470	0.0516	5.8
Tocainide	1600	0.0519	5.8
Lidocaine	2500	0.133	2.26

stereoisomers. These compounds have little difference in potency for tonic block in electrophysiological experiments. In contrast, at a stimulation frequency of 1 Hz, RAC 109 I ($K_{0.5}$ = 34 μM) is 14-fold more potent that RAC 109 II ($K_{0.5}$ = 490 μM) (28). Figure 6 illustrates the inhibition of [^{3}H]BTX-B binding by RAC 109 I and II. As with electrophysiological measurements, we also observe that RAC 109 I (K_D = 6.4 ± 2.4 μM) has substantially higher affinity than RAC 109 II (K_D = 56 ± 8 μM). Our estimates of K_D are lower than those from voltage clamp studies, yet the differences in potency between the two stereoisomers are similar. The observation that stereospecificity is involved in local anesthetic action, both in our binding assay and in electrophysiological experiments, is substantial evidence favoring action of these drugs at a specific receptor site on protein components of the sodium channel.

Yeh (28), in kinetic analysis of voltage clamp data using RAC 109 I and II, observed that the difference in potency between enantiomers was apparently due to a

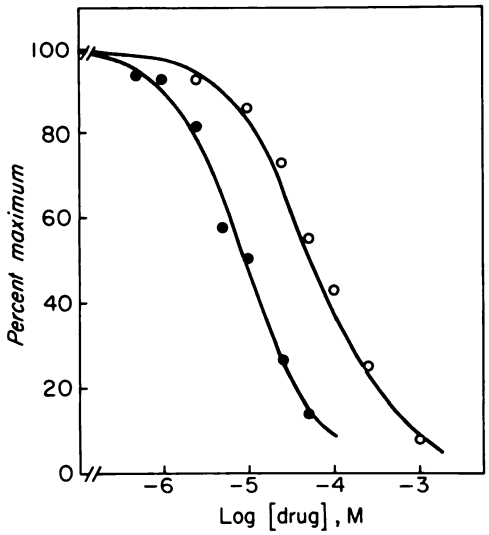


FIG. 6. *Effect of stereoisomers of local anesthetics on the binding of [^{3}H]BTX-B to synaptosomes*

Binding of 10 nM [^{3}H]BTX-B was measured in standard incubation medium in the presence of various concentrations of RAC 109 I (●) and RAC 109 II (○) as described under Experimental Procedures. The results represent data of a single experiment performed in duplicate in which RAC 109 I had $K_{0.5}$ = 34 μM and K_D = 8.7 μM while RAC 109 II had $K_{0.5}$ = 490 μM and K_D = 63 μM. The mean values of three comparable experiments were K_D = 6.4 ± 2.4 μM for RAC 109 I and K_D = 56 ± 8 μM for RAC 109 II.

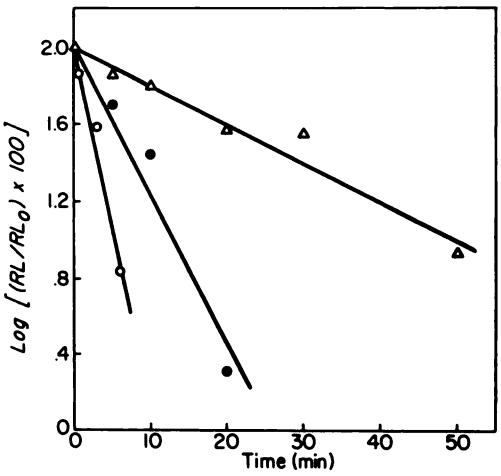


FIG. 7. *Dissociation of the [^{3}H]BTX-B/receptor complex in the presence of local anesthetic stereoisomers*

Synaptosomes were incubated in standard medium for 30 min with 10 nM [^{3}H]BTX-B and, at zero time, 200 μM aconitine in the absence (△) or presence of 60 μM RAC 109 I (●) or 600 μM RAC 109 II (○) was added. Owing to the limited solubility of aconitine, 1% ethanol was present throughout. At the indicated times, samples were removed and bound [^{3}H]BTX-B was determined as described under Experimental Procedures. The results presented represent data of a single experiment performed in duplicate. Average values of dissociation rate constants for three comparable experiments were 0.0181 ± 0.004 min^{-1} for aconitine alone, 0.058 ± 0.03 min^{-1} for aconitine plus RAC 109 I, and 0.218 ± 0.03 min^{-1} for aconitine plus RAC 109 II.

difference in rate of recovery from frequency-dependent block coupled with a difference in voltage dependence of cumulative block. Kinetic analysis of dissociation rates of [^{3}H]BTX-B binding in the presence of the RAC enantiomers at a concentration of about 10 K_D is depicted in Fig. 7. The dissociation rate constant for the [^{3}H]BTX-B complex is increased 9.3-fold in the presence of RAC 109 II and 4.3-fold in the presence of a comparable concentration of RAC 109 I. The difference in dissociation rate between these two enantiomers supports the concept of a stereospecific action on the [^{3}H]BTX-B receptor complex. The acceleration of dissociation by enantiomers in the presence of aconitine is not consistent with a competitive mechanism of action and suggests that the RAC compounds are indirect allosteric inhibitors of BTX binding.

DISCUSSION

Local anesthetics are amphipathic compounds that have a wide variety of effects on excitable membranes. However, their pharmacological efficacy derives from their reversible block of voltage-sensitive sodium channels in nerve. There is increasing evidence that this effect of local anesthetics results from actions at one or more discrete drug receptor sites on the sodium channel protein rather than a general perturbation of lipid bilayer structure. Quaternary amine local anesthetics act much more rapidly when applied from the intracellular side of the axonal membrane, indicating that their site of action is asymmetrically located and more accessible from the axoplasm (26, 29). Block of sodium currents is more rapid and more complete when sodium channels are

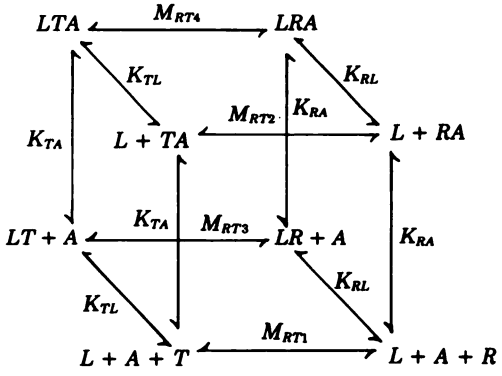
repetitively activated (24, 26, 27). This effect is quantitatively accounted for by a model which assumes that the drugs bind more rapidly to ion conducting state(s) of sodium channels and bind more tightly to inactivated state(s) (25). This mechanism assumes a protein receptor site whose conformation and accessibility change during channel activation and inactivation. Finally, certain enantiomeric local anesthetics have marked differences in potency in electrophysiological experiments (28). Our results extend these data by showing that the enantiomers RAC 109 I and II differ substantially in binding affinity as assessed by competitive block of [^{3}H]BTX-B binding. Taken together, these physiological and pharmacological data provide substantial support for interaction of amine local anesthetics with discrete receptor site(s) on voltage-sensitive sodium channels.

Several lines of evidence suggest that the action of local anesthetics may involve either direct or indirect interactions with neurotoxin receptor site 2 on the sodium channel. Like local anesthetics, BTX is lipophilic and has demonstrated frequency dependence in the development of its effects on ion movement (13). Electrophysiological experiments have provided indirect evidence for competitive interactions between BTX and certain local anesthetics (11, 12). In the absence of BTX, procaine blocks sodium channels at lower concentrations than those required to block BTX-activated channels, suggesting competitive interactions (12). Procaine and lidocaine prevent irreversible depolarization of skeletal muscle by batrachotoxin in a manner that suggests block of toxin binding (11). Ion flux studies in neuroblastoma cells have shown that the quaternary lidocaine derivative QX 572 and the electroneutral benzocaine are competitive inhibitors of BTX activation (14). Similar studies with a variety of antiarrhythmic drugs, including the local anesthetic lidocaine (10), revealed a competitive inhibition pattern for activation of channels by the full agonist BTX but a mixed inhibition pattern for channels activated by the partial agonist veratridine, suggeting that local anesthetics are allosteric competitive inhibitors of BTX action.

The current study provides additional evidence for involvement of neurotoxin receptor site 2 in the actions of local anesthetics. Local anesthetic inhibition of [^{3}H] BTX-B binding occurs at pharmacologically relevant concentrations and is well correlated with block of sodium currents by these drugs. Moreover, previous studies (9, 10) revealed that local anesthetics have little effect at sites 1 or 3 on the sodium channel. In view of the allosteric coupling between sites 2 and 3, it was important to rule out action at site 3. We observed little effect of tetracaine on ^{125}I-scorpion toxin binding but did observe an inhibition of BTX (1.25 μM)-enhanced scorpion toxin binding which was dose-dependent. The K_D value (1.7 μM) for inhibition of BTX-enhanced scorpion toxin binding by tetracaine correlates well with its K_D (1.2 μM) for inhibition of [^{3}H]BTX-B binding, suggesting that these effects occur at a single class of sites. The stereospecificity of enantiomeric local anesthetics in both equilibrium and kinetic experiments suggests that this interaction with the sodium channel involves specific interactions with protein components of the channel.

The mechanisms by which local anesthetic drugs affect neurotoxin binding were also examined in this study. Scatchard analysis revealed an increase in the K_D of BTX-B for its receptor in the presence of local anesthetics without a decrease in binding capacity and thus is consistent with a competitive mechanism of action. However, studies of the action of lidocaine in neuroblastoma cells indicated a competitive pattern of inhibition of persistent activation by the full agonist BTX but a mixed competitive/noncompetitive pattern of inhibition for the partial agonist veratridine (10). These results are expected for an allosteric inhibition mechanism (7). The kinetic studies of the effect of local anesthetics on the dissociation rate of the BTX/receptor complex confirm this finding, since the observed increase in dissociation rate constant in the presence of a saturating concentration of the competing ligand aconitine indicates an indirect allosteric competitive inhibition mechanism. Similar results are observed with the stereoisomers RAC 109 I and II.

Since local anesthetics appear to act by an allosteric mechanism of inhibition, further definition of their effect is possible (7, 16, 21). Previous work has shown that BTX and related lipid-soluble toxins cause persistent activation of sodium channels in neuroblastoma cells and synaptosomes by binding with high affinity to active states of the channel (7, 8, 21). If local anesthetics inhibit BTX binding by a similar allosteric mechanism, the binding of these drugs to R (active) states of sodium channels should have lower affinity than binding to T (nonconducting) states. Scheme I presents such a model,



SCHEME 1

where K_{TL} and K_{RL} are equilibrium dissociation constants for the binding of local anesthetics to the T and R states of the channel, respectively; K_{TA} and K_{RA} are the equilibrium dissociation constants for binding of alkaloid toxins such as BTX; and the M_{RT} values are equilibrium constants for the unimolecular conformational transition between the R and T states. In the presence of a local anesthetic (L) that binds tightly to the T state, channels are stabilized in that state. BTX and related alkaloid toxins (A) bind with higher affinity to R states of the channel.

Thus, $M_{RT2} < (M_{RT1}, M_{RT4}) < M_{RT3}$. From the derivation of Monod *et al.* (30), for the case of an allosteric system

TABLE 4

Computed dissociation constants for local anesthetics

Data taken from experiments like those of Figs. 1 and 7 were fit to Eq. 2 as described in the text to yield the values for K_{TL} and K_{RL}.

Drug	K_{TL}	K_{RL}	K_{RL}/K_{TL}
	M	*M*	
Tetracaine	1.1×10^{-6}	1.35×10^{-5}	12.9
Mepivacaine	1.19×10^{-4}	1.32×10^{-3}	11.1
W 36017	7.3×10^{-4}	6.67×10^{-3}	9.10
RAC 109 I	7.05×10^{-6}	1.7×10^{-4}	24.1
RAC 109 II	4.94×10^{-5}	6.82×10^{-4}	13.8

with no homotropic cooperativity ($n = 1$), we can derive an equation for the saturation function, Y_F, the fractional occupancy of sodium channel receptor sites by BTX-B, as a function of toxin concentration A, K_T, K_R, and M:

$$Y_F = \frac{M(A/K_T) + A/K_R}{M(1 + A/K_T) + 1 + A/K_R} \quad (1)$$

The effect of heterotropic allosteric inhibitors like the local anesthetics can be described as a change in M according to the equations

$$M' = M\left(\frac{1 + L/K_{TL}}{1 + L/K_{RL}}\right) \quad (2)$$

Here, the change in the allosteric constant results from the selective binding of the local anesthetics to the T state.

In order to estimate values of K_{TL} and K_{RL} for the local anesthetics, values of M' were calculated from the known values of M (788), A (10 nM), K_T (1.5×10^{-5} M), and K_R (1.1×10^{-10} M) derived from previous work (7, 21) and the measured values of Y'_F measured at each local anesthetic concentration using Eq. 1. Values of M' as a function of L were fit to Eq. 2 using an iterative least sum of squares method to give best estimates of K_{TL} and K_{RL}. These values are presented for selected local anesthetics in Table 4. Local anesthetics bind with at least 10-fold greater affinity to nonconducting states of sodium channels. This property of the local anesthetics is sufficient to account for their competitive inhibition of BTX binding and action and is consistent with the interpretation of the frequency dependence of local anesthetic action in terms of high-affinity binding to inactivated states of sodium channels (25). For compounds such as RAC 109 I which display marked differences in potency with repetitive stimulation (28), the difference in binding affinity to inactivated and activated states is also marked, with RAC 109 I binding to the inactivated state of the sodium channel with 24-fold greater affinity than to the activated state.

Taken together with previous studies, our results support the conclusion that local anesthetics bind to a discrete site(s) on sodium channels, which results in block of the channels and allosteric inhibition of BTX binding. Their mechanism of action involves, at least in part, high-affinity binding to inactivated states of sodium channels. It will be of considerable interest to examine the local anesthetic receptor sites on the sodium channel directly by measuring binding of radiolabeled local anesthetics.

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