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Batrachotoxin-Induced Depolarization and [^{3}H]Batrachotoxinin-A 20α-Benzoate Binding in a Vesicular Preparation from Guinea Pig Cerebral Cortex

Inhibition by Local Anesthetics

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SUMMARY

The sodium channel-specific agent batrachotoxin (BTX) has been shown to induce a time- and concentration-dependent depolarization of a vesicular preparation from guinea pig cerebral cortex. The $K_{0.5}$ for depolarization by BTX was 0.011 μM at 30 min. Membrane potential was determined by the equilibrium distribution of [^{3}H]triphenylmethylphosphonium ion. A series of seven local anesthetics was shown to inhibit BTX-induced depolarization competitively with K_i values ranging from 0.9 μM for dibucaine to 780 μM for lidocaine ethiodide. The specific binding of labeled batrachotoxinin-A 20α-benzoate ([^{3}H]BTX-B) to voltage-sensitive channels in vesicular preparations from mouse cerebral cortex in the presence of scorpion venom was measured and found to yield a range of K_d values from 25 to 30 nM and B_{max} values of 0.5 and 1.0 pmole/mg of protein; the same preparation from guinea pig cerebral cortex was found to yield K_d values from 13 to 56 nM and B_{max} values of 0.8–2.2 pmoles/mg of protein. A series of 14 local anesthetics was shown to inhibit the specific binding of [^{3}H]BTX-B with K_i values ranging from 0.6 μM for dibucaine to 400 μM for benzocaine. The rank order of potency of the local anesthetics as antagonists of [^{3}H]BTX-B binding was as follows: dibucaine > tetracaine > bupivacaine > diphenhydramine > piperocaine > cocaine > procaine > lidocaine > benzocaine. The quaternary local anesthetic dimethyl-di(phenylcarbamoylmethyl)ammonium chloride was comparable in potency to tetracaine. The rank order and relative potency of the local anesthetics tested in both paradigms were similar with the exception of lidocaine ethiodide, which was 18 times more potent as an inhibitor of binding of [^{3}H]BTX-B than it was as an inhibitor of BTX-elicited depolarization.

INTRODUCTION

Certain neurotoxins, of which BTX[3] is the most potent, are depolarizing agents which alter the voltage sensitivity of both activation and inactivation of the Na^+ channel by binding to a common site (1–4). A synthetic analogue, BTX-B, interacts with the same binding site and is equipotent with BTX. Radioactive BTX-B has proven useful in investigations of the binding characteristics of such sites (5–7).

Local anesthetics block voltage-dependent Na^+ channels in nerves, thereby eliciting a depression of the excitability of nerve fibers (8). Several mechanisms have been proposed for the action of local anesthetics (9–11). Such mechanisms must accommodate the remarkable structural diversity of local anesthetics which include permanently charged, cationic, ionizable, and neutral compounds. The simplest mechanism, the one-site model, suggests that local anesthetics, both charged and uncharged, inhibit Na^+ conductance by binding at a single site associated with the voltage-dependent Na^+ channel. The wide variations in potency and duration of action seen with different local anesthetics in such a mechanism would result from different rates of access to this binding site as well as their intrinsic affinity for the site (12).

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[3] The abbreviations used are: BTX, batrachotoxin; BTX-B, batrachotoxinin-A 20α-benzoate; $TPMP^+$, triphenylmethylphosphonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QX-572, dimethyl-di(phenylcarbamoylmethyl)ammonium chloride.

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Since there appears to be a mutual antagonism between actions of local anesthetics and BTX (2, 3, 13–15), it was felt that [^{3}H]BTX-B might provide a specific probe for measuring possible interactions of local anesthetics with the BTX-binding site associated with Na^+ channels.

One model system for studying such interactions makes use of vesicular elements from brain which maintain a transmembrane potential of −58 to −78 mV (16). This membrane potential can be determined by measurement of the equilibrium distribution of the lipophilic cation [^{3}H]TPMP$^+$. BTX induces a concentration- and time-dependent depolarization in this vesicular preparation which is blocked by tetrodotoxin and is potentiated by scorpion toxin and sea anemone toxin-II (16). The effects of seven local anesthetics on both BTX-induced depolarization and on binding of [^{3}H]BTX-B were compared in guinea pig cerebral cortical vesicular preparations.

EXPERIMENTAL PROCEDURES

Materials. Scorpion venom of *Leiurus quinquestriatus* was obtained from Sigma Chemical Company (St. Louis, Mo.). Tetrodotoxin was from Calbiochem Corporation (Rockville, Md.). Veratridine was purchased from Aldrich Chemical Company (Milwaukee, Wisc.).[4] BTX was obtained from Colombian poison-dart frogs (17). Grayanotoxin I was provided by Dr. W. M. Tallant, Northern Regional Research Center (Peoria, Ill.). The local anesthetics used were obtained from a variety of sources: dibucaine·HCl from Ciba Pharmaceutical Company (Summit, N. J.); diphenhydramine·HCl and tetracaine·HCl were from Sigma Chemical Company; piperocaine·HCl from Eli Lilly and Company (Indianapolis, Ind.); cocaine·HCl was from Merck & Company Inc. (Rahway, N. J.); bupivacaine was from Sterling-Winthop Research Institute (Rensselaer, N. Y.). Dr. L.-Y. M. Huang, of the Laboratory of Biophysics, National Institute of Neurological and Communicative Diseases and Stroke, kindly supplied us with procaine·HCl, lidocaine·HCl, benzocaine, QX-572, and lidocaine ethiodide. BTX-B and [^{3}H]BTX-B, with a specific activity of 14 Ci/mmole, was prepared as described (5). TPMP$^+$, with a specific activity of 63 mCi/mmole, was prepared as described (16). Methiodides of piperocaine, bupivacaine, and diphenhydramine were prepared by standard methods (see ref. 18). Other compounds were from standard commercial sources.

Methods. The preparation containing the vesicular elements was obtained from slices of rodent cerebral cortex by a modification (19) of the original procedure of Chasin *et al.* (20). The biochemical and morphological characteristics of the components of this preparation have been described (16, 19). The major components appear to be resealed postsynaptic elements and attached presynaptic endings. Briefly stated, the rodent brain (male Hartley guinea pig or Swiss-Webster mouse as indicated) was removed and placed immediately in either Krebs-Ringer bicarbonate-glucose buffer (pH 7.4) or Hepes buffer (pH 7.4). The cerebral cortex was sliced away by hand and placed in a conical, loose-fitting, glass-glass homogenizer. The tissue was homogenized by hand (10–12 strokes) in 4 ml of buffer, diluted with an additional 4 ml of buffer, and centrifuged at 1000 × *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 10 ml of fresh buffer for assay.

The use of the lipophilic cation [^{3}H]TPMP$^+$ as a measure of membrane potential has been described previously (16). Briefly stated, 100 μl of the above vesicular preparation were incubated in Krebs-Ringer bicarbonate-glucose buffer (pH 7.4) containing [^{3}H]TPMP$^+$ (86 μM). Incubations were carried out for various times, generally for 10–30 min at 37° in 12 × 75 mm tubes. The incubations were terminated by diluting with 4 ml of ice-cold phosphate-buffered saline (pH 7.2) and quickly filtering through a Millipore Cellotate filter (Millipore EH, 0.5 μM), followed by a second wash with 4 ml of buffered saline. Filters were dissolved and counted in Filtron-X scintillation solution (National Diagnostics, Somerville, N. J.). Uptake values were corrected for background binding of [^{3}H]TPMP$^+$ to the filter in the absence of the vesicular preparation.

Binding of [^{3}H]BTX-B was determined by a modification of the technique described by Catterall *et al.* (6). The vesicular preparation was placed in binding medium containing the following: 130 mM choline chloride, 50 mM Hepes buffer [adjusted to pH 7.4 with Tris (approximately 23 mM Tris base)], 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl. After the initial sedimentation, the pellet was washed with 10 ml of binding medium and resuspended in the same amount for use in binding reactions. Incubations were carried out in a total volume of 250 μl containing 1 μM tetrodotoxin, 0.03 mg of scorpion venom, 50 nM [^{3}H]BTX-B (unless otherwise indicated), and approximately 400 μg of protein of the vesicular preparation. Maximal specific binding was seen at 30 min when incubations were carried out at 37°. Binding reactions were terminated by diluting the incubation mixture with 3 ml of wash medium and collecting under vacuum on a glass-fiber filter (Whatman GF/C). The filters were then washed three times with 3 ml of wash medium. Wash medium consisted of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$. Filters were placed in scintillation vials with 10 ml of Hydrofluor (National Diagnostics), and the tritium content was measured by scintillation spectroscopy with an efficiency of 43%. Protein concentrations were determined (after washing samples with phosphate-buffered saline or by making the appropriate correction for the presence of the Hepes buffer) by the method of Lowry *et al.* (21), adapted for a Technicon autoanalyzer.

Nonspecific binding was determined by incubation of [^{3}H]BTX-B in the presence of 300 μM veratridine or 2 μM nonradioactive BTX-B as indicated. The specific binding was calculated by subtracting nonspecific from total binding values. Nonspecific binding was 24 ± 3% (mean ± standard error of the mean) of total binding. The radiopurity of [^{3}H]BTX-B was monitored by thin-layer chromotography (Silica gel G, Analtech) with authentic BTX-B as carrier, in chloroform/methanol, 20:1. BTX-B (R_F = 0.5) was visualized with iodine vapor. [^{3}H]BTX-B, stored in methanol at 4°, was stable for

[4] Veratridine is no longer commercially available. Further supplies have been isolated from mixed veratrine alkaloids (G. B. B.).

approximately 3 years. When radioimpurities began to appear, it became necessary to repurify the [^{3}H]BTX-B. This was accomplished with the same thin-layer system which separated the major contaminant ($R_F = 0.6$) from carrier-free [^{3}H]BTX-B. It was essential to remove the area corresponding to [^{3}H]BTX-B immediately upon completion of the chromatography into methanol. No attempt was made to characterize the impurities. The radioimpurities did not interfere with specific binding at concentrations less than 50% of the total tritium. The nonspecific binding decreased from 24% to 14% when radiopure [^{3}H]BTX-B was used.

RESULTS

Inhibition of uptake of TPMP$^+$ by local anesthetics. It was observed that local anesthetics, at relatively high concentrations, reduced the intravesicular accumulation of [^{3}H]TPMP$^+$. This appeared to result from a direct effect on equilibrium distribution of [^{3}H]TPMP$^+$, with the local anesthetics acting as a competing lipophilic cation. It was demonstrated that the lipophilic cation, tetraphenylphosphonium will also effectively reduce accumulation of [^{3}H]TPMP$^+$ (data not shown). To dissociate the direct effects of local anesthetics on uptake of the indicator probe [^{3}H]TPMP$^+$ from the inhibition of BTX-induced depolarization, it was necessary to determine the dose-response relationships for this apparent competition or displacement phenomena. The concentrations of local anesthetics needed to produce the apparent direct competition of [^{3}H]TPMP$^+$ uptake were in all cases 50- to 100-fold greater than the concentrations needed to block BTX-induced depolarization. Dibucaine had an IC_{50} of 71 μM with respect to direct competition of uptake of [^{3}H]TPMP$^+$, followed by diphenhydramine with an IC_{50} of 182 μM. QX-572, tetracaine, piperocaine, and cocaine had IC_{50} values of 210, 320, 630, and 1550 μM, respectively. The remaining local anesthetics tested (see Table 1), benzocaine and lidocaine ethiodide, yielded only minimal competition versus [^{3}H]TPMP$^+$ uptake at concentrations up to 2000 μM. Thus it was clearly evident that the ability of local anesthetics to penetrate the plasma membrane and reduce accumulation of [^{3}H]TPMP$^+$ varied over a wide range depending upon the structure of the local anesthetic. It should be noted that quaternary amines (like QX-572) reduced the accumulation of [^{3}H]TPMP$^+$, suggesting that a quaternary amine structure does not preclude competition for passage through the lipid bilayer. In all subsequent studies versus BTX-induced depolarization, the concentrations of local anesthetics used were well below the concentrations which yielded significant direct competition of [^{3}H]TPMP$^+$ uptake.

BTX-induced depolarization. The Na^+ channel specific alkaloid, BTX, induces a concentration- and time-dependent depolarization of the transmembrane potential in the vesicular preparation based on the reduction in uptake of [^{3}H]TPMP. The equilibrium distribution of [^{3}H]TPMP has previously been shown to be a sensitive measure of the apparent membrane potential across the membranes of the vesicular components of this preparation (16). This BTX-induced depolarization, as previously described (16), represents approximately 72% of the apparent resting membrane potential of −68 mV maintained in the vesicular preparation. This BTX-sensitive component, defined as the neuronal membrane potential, was estimated by subtracting from experimental values the vesicular [^{3}H]TPMP$^+$ levels remaining in the presence of supramaximal concentrations of BTX. The BTX-induced depolarization as determined by the reduction in the uptake of [^{3}H]TPMP in this neuronal compartment after a 30-min exposure to BTX is shown in Fig. 1. When this dose-response curve is expressed as a double-reciprocal plot (*inset*, Fig. 1) it yields a linear plot, giving an apparent $K_{0.5}$ for BTX-induced depolarization of 11 ± 2 nM. It should be emphasized that the $K_{0.5}$ for BTX-induced depolarization, which is at least 3-fold lower than the binding constant for [^{3}H]BTX-B (see below), reflects events leading to a maximal depolarization of membrane potential. Maximal depolarization will occur when BTX binds to only a small fraction of the total number of Na^+ channels. The $K_{0.5}$ values obtained for BTX in this manner decreased rapidly over the first 10–15 min of exposure and then slowly declined over the next 90 min (data not shown). A depolarization period of 30 min was selected because it represented the major phase of depolarization without compromising the maintenance of the membrane potential in control vesicular preparations.

It should be emphasized that the linear form of the double-reciprocal plot of the relationship between the concentration of BTX and the percentage depolarization obtained under the present conditions may be entirely fortuitous. Certainly the binding and subsequent effect of BTX on membrane potential is the resultant of a complicated multicomponent system which is modulated by changes in membrane potential. This caution must

TABLE 1

Inhibition of BTX-induced depolarization of vesicular preparations from guinea pig cerebral cortex by local anesthetics

The inhibition of BTX-induced depolarization of vesicular preparations by local anesthetics was determined by measuring the equilibrium distribution of [^{3}H]TPMP$^+$ (86 μM) at 30 min as described under Methods. The displacement to the right of dose-response curves for BTX at concentrations ranging from 1 to 100 nM were determined at three different concentrations of local anesthetics. The contribution of the BTX-insensitive or non-neuronal fraction of the membrane potential (23%) was determined in the presence of a supermaximal concentration of BTX (2 μM), and the equilibrium distribution of [^{3}H]TPMP$^+$ was, in each case, corrected for this difference. The inhibition constant (K_i) for each local anesthetic was estimated by the method of least squares from replots of the apparent $K_{0.5}$ values and from Dixon plots as shown in Fig. 2A and B. In all cases the type of inhibition at the concentrations of local anesthetic used appeared to be competitive in nature. The values are the means of two determinations performed in triplicate.

Local anesthetic	Inhibition of BTX induced depolarization (K_i)
	μM
Dibucaine	0.94
Tetracaine	3.3
QX-572	3.8
Diphenhydramine	10
Piperocaine	19
Cocaine	26
Lidocaine ethiodide	780

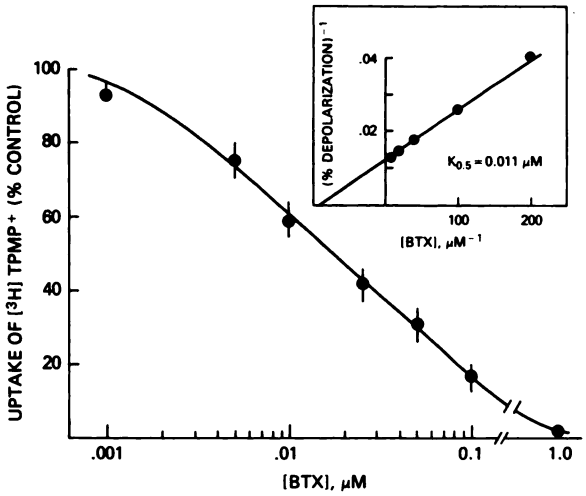

FIG. 1. *Inhibition of the uptake of [^{3}H]TPMP$^+$ in a vesicular preparation from guinea pig cortex by BTX*

The equilibrium distribution of [^{3}H]TPMP$^+$ (86 μM) was measured in vesicular preparations exposed to increasing concentrations of BTX for 30 min at 37°. The non-BTX-sensitive uptake of [^{3}H]TPMP$^+$, determined in parallel incubations with an excess of BTX (2 μM), has been subtracted. Membrane potential is expressed as percentage of [^{3}H]TPMP$^+$ uptake in the absence of BTX. Data points represent the mean ± standard error of the mean for nine separate experiments. A double-reciprocal plot of the data presented in the *inset* shows the intercept on the horizontal axis (−90.9) or a $K_{0.5}$ of 0.011 μM for BTX.

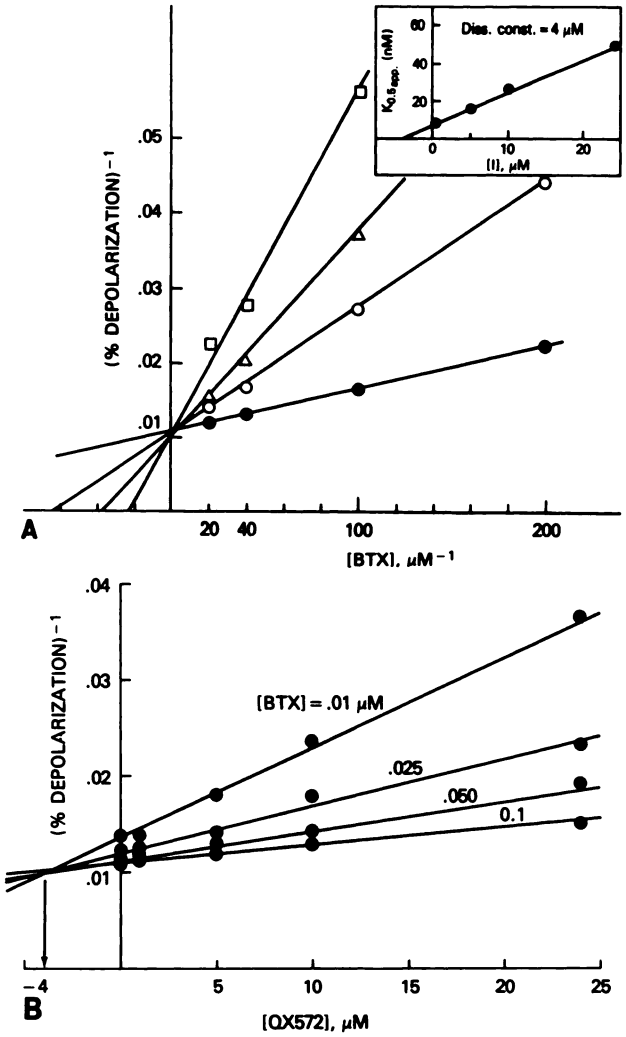

FIG. 2. *Competitive inhibition of BTX-induced depolarization by the local anesthetic QX-572*

A. Double-reciprocal plot. The equilibrium distribution of [^{3}H]TPMP$^+$ in 30 min at 37° was determined at increasing BTX concentrations from 5 to 50 nM in the presence of 0 (●), 5 (○), 10 (△), and 25 (□) μM QX-572. The data points represent the mean of three determinations and are plotted in a double-reciprocal form to a constant value for the vertical intercept (V_{max}) and a progressively decreasing horizontal intercept ($-1/K_{0.5}$ app). A replot of the apparent values for $K_{0.5}$ versus the concentration of QX-572 yielded a straight line, with the horizontal intercept indicating the inhibitor constant for QX-572 of 4 μM. Similar analyses were carried out with three different concentrations selected to yield approximately 25%, 50%, and 75% inhibition for each of the local anesthetics listed in Table 1.

B. Dixon plot. The data were replotted according to Dixon, plotting the reciprocal of the percentage depolarization against the concentration of local anesthetic for each concentration of BTX. The data points were fit by linear regression lines and intersected to the left of the vertical axis at a point equal to 0.01, or 100% depolarization. The value of the inhibition constant (K_i = 4 μM) is given by the point below the intersect on the horizontal axis.

also be applied to the linear plot obtained for the inhibition of BTX-induced depolarization by local anesthetics (see below).

Inhibition of BTX-induced depolarization by local anesthetics. The BTX-induced depolarization of the vesicular preparation was inhibited by a variety of both the tertiary and quarternary forms of local anesthetics. The effect of local anesthetics was to shift the dose-response curve for BTX-induced depolarization (Fig. 1) to the right. The relative potency of this inhibition was estimated by plotting the dose-response curves in a double-reciprocal manner. A representative example is shown in Fig. 2A, for QX-572. The double-reciprocal plot indicated no change in V_{max} with increasing concentrations of local anesthetic and a progressive increase in the apparent $K_{0.5}$ value, suggesting competitive inhibition. Replots of the apparent $K_{0.5}$ values for BTX in the presence of the local anesthetic (*inset*, Fig. 2A) yielded the apparent dissociation constant (see Fig. 2A). When the reciprocal of the BTX-induced depolarization at a given BTX concentration was plotted versus the concentration of local anesthetic in a manner according to Dixon (see ref. 22), the slope of the plots decreased with local anesthetic concentration, intersecting to the left of the vertical axis with a horizontal line drawn at level of $1/V_{max}$. A representative example is shown in Fig. 2B for the quarternary local anesthetic, QX-572. Again there was an apparent increase in the $K_{0.5}$ value and no change in the V_{max}, characteristic of competitive inhibition. The K_i values for the local anesthetics, estimated from similar Dixon plots, are listed in Table 1. Attempts to obtain acceptable kinetic data for competitive inhibition of depolarization by benzocaine were unsuccessful. However, estimates of the inhibition constant were approximately 1 mM. In all cases, the concentrations of local anesthetics utilized in these kinetic evaluations were below the concentrations which competed directly with [^{3}H]TPMP$^+$ uptake.

The inhibition of BTX-induced depolarization was ex-

amined with one local anesthetic, cocaine, over a range of concentrations that included high concentrations which directly affect uptake of [^{3}H]TPMP$^+$. Double-reciprocal plots for the dose-response of BTX-induced depolarization in the presence of cocaine at 25, 50, and 100 μM yielded a competitive inhibition pattern with a K_i for cocaine of 26 μM. At concentrations of 300 μM and higher the double-reciprocal plot began to shift progressively to the parallel pattern characteristic of uncompetitive inhibition (data not shown). Thus it was evident that the distribution of [^{3}H]TPMP$^+$ is not a measure of membrane potential at concentrations of local anesthetics which significantly interfere with equilibrium distribution of the probe. However, in all cases, the competitive inhibition of BTX-induced depolarization occurred at concentrations which did not affect the equilibrium distribution of [^{3}H]TPMP$^+$.

Specific binding of [^{3}H]BTX-B. [^{3}H]BTX-B had been shown to be a specific ligand for sites on voltage-dependent Na$^+$ channels in synaptosomal membranes from mouse and rat brain (5, 6). In the synaptosomal preparation, the affinity of [^{3}H]BTX-B for its binding site could be increased by a factor of approximately 15-fold in the presence of scorpion toxin (6). A similar scorpion venom-induced increase in the binding affinity of [^{3}H]BTX-B was observed with the vesicular preparation from guinea pig cerebral cortex (data not shown). This increased affinity allows effective use of a filtration assay with a favorable ratio of specific to nonspecific binding, and for this reason *Leiurus* scorpion venom, at a concentration of approximately 0.12 mg/ml, has been included in all experiments reported here. In addition it was observed that inclusion of tetrodotoxin up to a saturating concentration of 1 μM increased the specific binding by approximately 7%. Therefore, unless specifically noted, 1 μM tetrodotoxin was included in all experiments. The effect of tetrodotoxin was presumably due to prevention of depolarization through exclusion of extravesicular Na$^+$ ions still present in the vesicular preparation. The effects of scorpion toxin on the binding of BTX to its sites is known to be strongly dependent on the maintenance of membrane potential (6). The displacement of specifically bound [^{3}H]BTX-B by increasing concentrations of unlabeled BTX-B in the vesicular preparations from both mouse and guinea pig is illustrated in Fig. 3. The data are consonant with a single saturable and noninteracting binding site for BTX-B, with K_d values of 32 and 56 nM for mouse and guinea pig, respectively. The same data treated in a Scatchard analysis yield a straight line corresponding to K_d = 30 nM and B_{max} = 1.0 pmole/mg of protein for mouse and K_d = 56 nM and B_{max} = 2.2 μmoles/mg of protein for guinea pig (*inset*, Fig. 3). The K_d values obtained with mouse microsacs ranged from 25 to 32 nM and B_{max} values from 0.5 to 1.0 pmole/mg of protein; with guinea pig microsacs the K_d values ranged from 13 to 56 nM (mean ± standard error of the mean, 34.8 ± 4.6 nM; N = 10) and B_{max} values from 0.8 to 2.3 μmoles/mg of protein (mean ± standard error of the mean, 1.5 ± 0.1 nM; N = 9).

Several attempts were made to determine the K_d val-

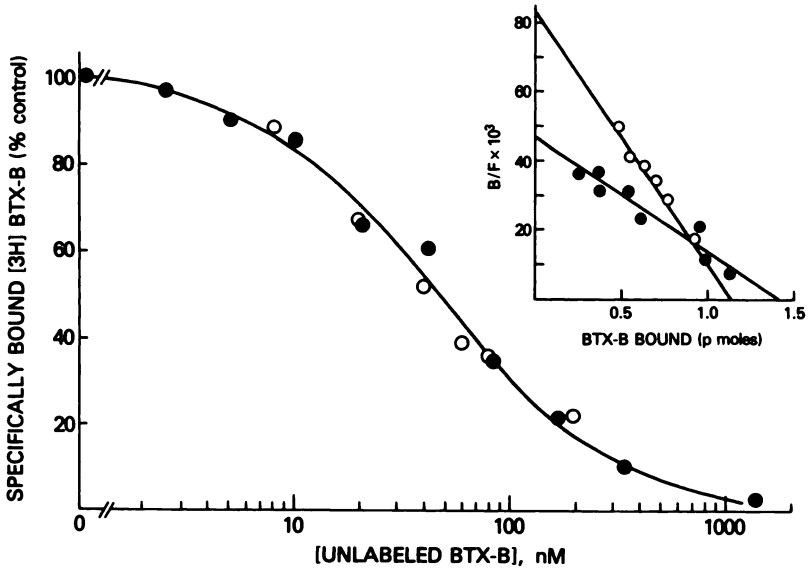

FIG. 3. *Inhibition of binding of [^{3}H]BTX-B to vesicular preparations from the cerebral cortex of mouse and guinea pig by unlabeled BTX-B*

Mouse (●): Binding of [^{3}H]BTX-B (8 nM) to the vesicular preparation (1.4 mg of protein per assay tube) was determined at 37° in the presence of increasing concentrations of unlabeled BTX-B. Tetrodotoxin was not included in the incubation. Nonspecific binding, determined from parallel incubations containing BTX-B (2 μM), has been subtracted. Data points represent means of duplicate determinations. Half-maximal inhibition of specific binding was found at 78 nM unlabeled BTX-B. The total specifically bound BTX-B (unlabeled and labeled) at each concentration is presented as a Scatchard plot (*inset*). The data were fit by a linear regression line with slope corresponding to K_d = 30 nM and an intercept on the abscissa corresponding to a B_{max} of 1.4 pmoles/assay (1.0 pmoles/mg of protein).

Guinea pig (○): Binding of [^{3}H]BTX-B (43 nM) to the vesicular preparation (0.5 mg/assay tube) was determined in the presence of increasing concentrations of unlabeled BTX-B as described under Methods. Half-maximal inhibition of specific binding was found at 78 nM unlabeled BTX-B. The total specifically bound BTX-B is presented as a Scatchard plot (*inset*). The data were fit by a linear regression line with a slope corresponding to K_d = 56 and an intercept on the abscissa corresponding to a B_{max} of 1.1 pmoles/assay (2.2 pmoles/mg of protein).

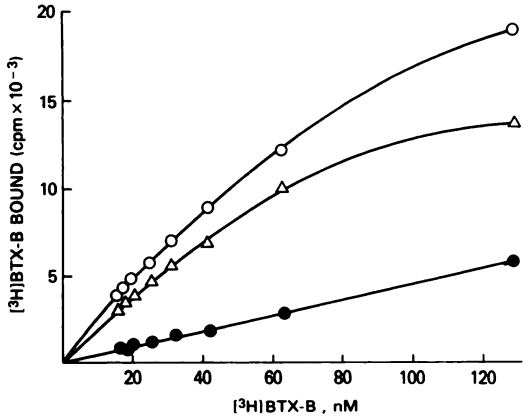

FIG. 4. *Binding of [^{3}H]BTX-B to the vesicular preparation from guinea pig cerebral cortex*

An example of the specific binding of [^{3}H]BTX-B with the preparation from guinea pig (0.5 mg of protein per assay tube) with 15–129 nM [^{3}H]BTX-B for 30 min at 37° in the presence of tetrodotoxin (1 μM) and scorpion venom (0.03 mg). Each data point represents the mean of three determinations. The linear curve (●) represents the nonspecific binding obtained from parallel incubations in the presence of veratridine (300 μM) and is 23% of the total binding (○). The difference (△) represents the specific binding (76%).

ues with increasing concentrations of [^{3}H]BTX-B alone. Figure 4 illustrates a typical binding experiment with [^{3}H]BTX-B alone yielding specific binding of 76–78% with guinea pig vesicular preparations. Treating these data in a double-reciprocal manner or as a Scatchard plot yields a linear relationship in both cases corresponding to an apparent K_d = 113 nM and B_{max} = 3.8 pmoles/mg of protein. The results of seven separate attempts in this manner yielded a mean apparent K_d = 134 ± SD 46 nM and B_{max} = 3.6 ± SD 0.7 pmole/mg of protein. The nature of this discrepancy between the K_d values obtained by isotope dilution with unlabeled BTX-B and with [^{3}H]BTX-B alone is unresolved at present. For comparison, the concentration of binding sites for [^{3}H]BTX-B determined in rat synaptosomes (6) is 2 pmoles/mg of protein, with a K_d of approximately 80 nM.

The displacement of specifically bound [^{3}H]BTX-B from mouse vesicular preparations by BTX, veratridine, and grayanotoxin is shown in Fig. 5. The apparent K_i values for these compounds are 0.035, 8, and 100 μM, respectively. The displacement of [^{3}H]BTX-B by BTX from guinea pig vesicular preparations yielded an apparent K_i of 0.075 μM for batrachotoxin. These values are in agreement with the respective $K_{0.5}$ values of 0.052, 15, and 92 μM obtained by Catterall (1) for the activation of voltage-sensitive Na^+ channels in neuroblastoma cells in the presence of scorpion toxin and with the K_i values of BTX (0.05 μM) and veratridine (7 μM) for antagonism of binding of [^{3}H]BTX-B to rat brain synaptosomes in the presence of scorpion toxin (6).

Inhibition of [^{3}H]BTX-B binding by local anesthetics. The inhibition of binding of [^{3}H]BTX-B to the vesicular preparation from guinea pig cortex by various local anesthetics is shown in Fig. 6. The slopes of the displacement curves are nearly parallel in all cases except for that of the least potent anesthetic tested, benzocaine. The potency of the inhibition for the displacement of [^{3}H]BTX-B (IC_{50}) was estimated from these curves (Table 2). The ability of local anesthetics to displace [^{3}H]BTX-B decreased from the most effective, dibucaine (IC_{50} = 1.4 μM), in a continuum to the least effective, lidocaine and benzocaine (IC_{50} = 240 and 910 μM, respectively). The rank order of the inhibitory potency for displacement of [^{3}H]BTX-B (Table 2) very closely parallels the potency of local anesthetics with respect to inhibition of BTX-elicited depolarization of the vesicular preparations (Table 1). The only exception to the rank order correlation of the compounds tested was exhibited by lidocaine ethiodide, which was 18 times less potent as an inhibitor of BTX-induced depolarization (K_i = 780

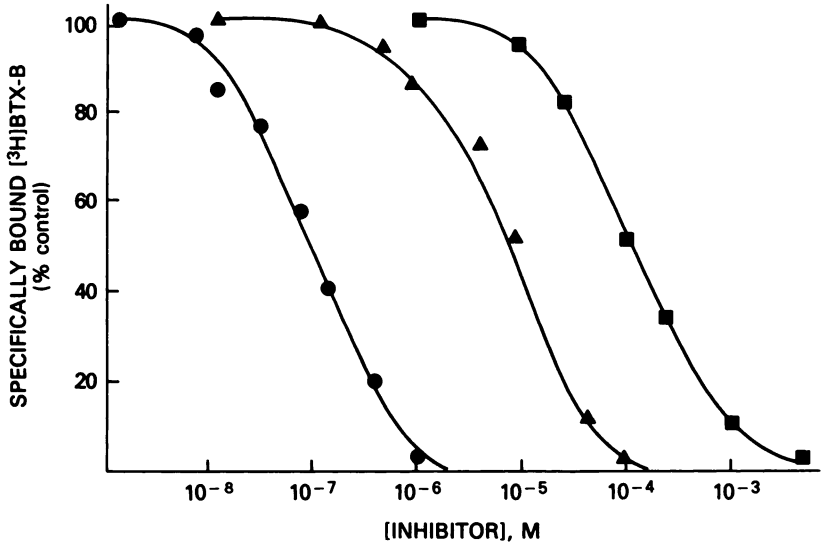

FIG. 5. *Inhibition of binding of [^{3}H]BTX-B by BTX, veratridine, and grayanotoxin I*

The binding of [^{3}H]BTX-B (40 nM) to a vesicular preparation from mouse cerebral cortex at 37° was determined in the presence of increasing concentrations of BTX (●), veratridine (▲), and grayanotoxin I (■). Nonspecific binding of [^{3}H]BTX-B, determined in parallel incubations containing unlabeled BTX-B (1 μM), has been subtracted. Data points represent the means of duplicate determinations.

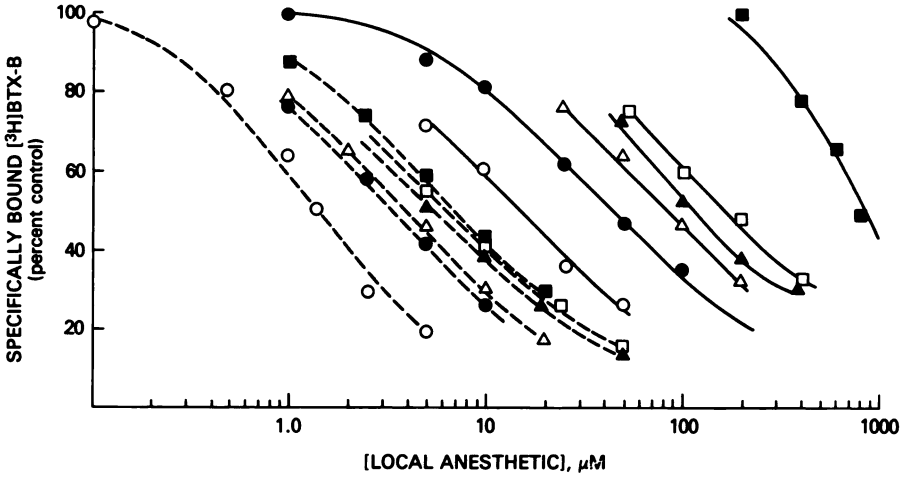

FIG. 6. *Inhibition of binding [^{3}H]BTX-B to a vesicular preparation from guinea pig cerebral cortex by local anesthetics*

The binding of [^{3}H]BTX-B (43 nM) in the presence of increasing concentrations of several local anesthetics is plotted as percentage of [^{3}H]BTX-B binding in the absence of local anesthetic (percentage control) versus the concentration of local anesthetic. Each data point represents the mean of three measurements. The K_i values are presented in Table 2, calculated from the concentration of local anesthetic yielding a 50% reduction in specific binding. From *left* to *right* the anesthetics tested were dibucaine (○- - -○), tetracaine (●- - -●), QX-572 (△- - -△), bupivicaine methiodide (▲- - -▲), bupivicaine (□- - -□), diphenhydramine (■- - -■), piperocaine (○——○), cocaine (●——●), lidocaine ethiodide (△——△), procaine (▲——▲), lidocaine (□——□), and benzocaine (■——■).

μM) than it was as an inhibitor of the binding of [^{3}H]BTX-B (K_i = 43 μM). The inhibition of BTX-induced depolarization by lidocaine ethiodide did appear to be competitive in nature (data not shown). Quaternization had greatly reduced the potency of lidocaine versus BTX-elicited depolarization, while actually increasing potency versus binding of [^{3}H]BTX-B. However, quaternization of local anesthetics did not lead to consistent changes in the potency as antagonists of binding of [^{3}H]BTX-B, as compared with the potency of the structurally related tertiary amines. Thus quaternization of bupivicaine to bupivicaine methiodide and lidocaine to lidocaine ethiodide resulted in minimal changes in potency whereas the methiodides of diphenhydramine and piperocaine were 8 and 28 times less potent than their respective tertiary amines (see Table 2). The tertiary amine analogue for QX-572 was not available. Apparently the presence of a quaternary nitrogen can increase, decrease, or have no effect on the potency of local anesthetics as antagonists of binding of [^{3}H]BTX-B.

TABLE 2

Inhibition of binding of [^{3}H]BTX-B from vesicular preparations from guinea pig cerebral cortex by local anesthetics

The potencies of the local anesthetics (IC_{50}) were determined from curves of the displacement of [^{3}H]BTX-B in the presence of scorpion venom (0.12 mg/ml) and tetrodotoxin (1.0 μM) in 30 min from vesicular preparations as described under Methods. The values for nonspecific binding of [^{3}H]BTX-B in the presence of veratridine (300 μM) were subtracted from the total binding. Specific binding of [^{3}H]BTX-B was 76%. Each value represents the average of two or three determinations performed in triplicate. The inhibition constants (K_i) were calculated from the IC_{50} values as described in the text, using the mean K_d value of 35 nM.

Local anesthetic	Inhibition of binding of [^{3}H]BTX-B	
	IC_{50}	K_i
	μM	μM
Dibucaine	1.4	0.63
Tetracaine	3.4	1.5
QX-572	3.9	1.7
Bupivacaine methiodide	4.8	2.1
Bupivacaine	5.4	2.4
Diphenhydramine	6.0	2.7
Piperocaine	13	5.8
Cocaine	49	22
Diphenhydramine methiodide	64	29
Lidocaine ethiodide	97	43
Procaine	110	49
Lidocaine	240	110
Piperocaine methiodide	360	160
Benzocaine	910	410

In order to verify that local anesthetics competitively inhibit the specific binding of [^{3}H]BTX-B, the binding data in the presence and absence of representative local anesthetics were subjected to Scatchard analysis. As shown in Fig. 7A for dibucaine and Fig. 7B for QX-572, the effect of these local anesthetics was to change the apparent K_d for binding of [^{3}H]BTX-B with little or no change in B_{max}. In the presence of dibucaine, 0.5 and 1.0 μM, the apparent K_d for the specific binding of [^{3}H]BTX-B increased from 38 nM for BTX-B alone to 82 and 120 nM, respectively, while the V_{max} decreased from 1.4 to 1.3 and 1.2 pmoles bound per milligram of protein (Fig. 7A). Similarly, in the presence of QX-572 (5 μM), the apparent K_d for BTX-B increased from 50 to 192 nM while the measured V_{max} increased slightly from 1.3 to 1.5 pmoles bound per milligram of protein.

The apparent competitive nature of the effect of local anesthetics permits the calculation of the inhibitor constant, K_i, from the IC_{50} values for the displacement of [^{3}H]BTX-B according to the following relationship: $K_i = IC_{50}/(1 + L/K_d)$, where L = the concentration of [^{3}H]BTX-B (0.043 μM) and the mean K_d value for [^{3}H]BTX-B is 0.035 μM (under our conditions, $K_i = (IC_{50}) \times (0.448)$. The K_i values calculated in this manner are listed in

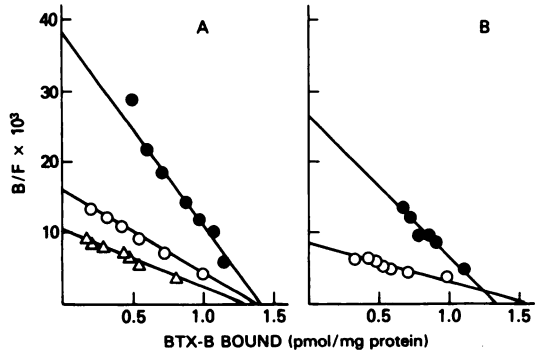

FIG. 7. *Scatchard analysis of the specific binding of [³H]BTX-B to a vesicular preparation from guinea pig cerebral cortex in the presence and absence of representative local anesthetics*

A, Dibucaine, 0.5 μM (○) and 1.25 μM (△); B, QX-572, 0.4 μM (○); BTX-B alone (●).

Table 2. It should be clearly emphasized that the inhibition constants for local anesthetics with regard to binding of [³H]BTX-B were obtained under quite different conditions than the values obtained for the inhibition of BTX-induced depolarization. The binding study was carried out in the presence of tetrodotoxin and scorpion venom and in the absence of external Na^+, whereas investigation of BTX-induced depolarization was carried out in the presence of physiological concentrations of external Na^+ (147 mM) and no tetrodotoxin or scorpion venom. It is of interest, however, that even with these differences the ratio of the inhibition constants derived from Dixon plots of the inhibition of BTX-induced depolarization and the values calculated from the displacement of [³H]BTX-B ranged from 1.2 to 3.7. The ratios were as follows: dibucaine, 1.5; tetracaine, 2.2; QX-572, 2.2; diphenhydramine, 3.7; piperocaine, 3.3; cocaine, 1.2; and lidocaine ethiodide, 18. The only exception was lidocaine ethiodide, which gave a ratio of 18. The apparent correspondence of the order of these two separate measures of the interaction of local anesthetics and with BTX-induced changes in membrane potential and the affinity of BTX-B for the BTX-binding site on the Na^+ channel suggests that most local anesthetics act by a single mechanism which is competitive or mutually exclusive with the binding of BTX to a specific binding site associated with the Na^+ channel.

DISCUSSION

The delineation of mechanism(s) of action of local anesthetics have in the past relied upon the measurement of various parameters of electrically excitable membrane systems, primarily changes in membrane potential or ion flux, and structure-activity evaluations based upon the physicochemical properties of local anesthetics (8). Such investigations have demonstrated that local anesthetics depress conduction in nerve fibers and other electrically excitable tissues by blocking Na^+ channels (23–25). The interaction of amine local anesthetics with the Na^+ channel is complex and has been shown to be dependent upon both the frequency and duration of Na^+ channel activation and inactivation (9–12, 25). These observations led to the proposal that local anesthetics bind to a specific site associated with the gating mechanism of Na^+ channels and that the rate and affinity of this binding of charged local anesthetics is increased in direct proportion to the number of Na^+ channels present in the open or conducting form (9–12, 26).

The neurotoxic alkaloid BTX binds to a specific site in the Na^+ channel. This binding alters the Na^+ channel, resulting in a persistence of the open or conducting form and thus profound membrane depolarization (3). The rate and extent of BTX action has been shown to be stimulus-dependent and is markedly enhanced by an allosteric mechanism consequent to the binding of polypeptides such as scorpion toxin or sea anemone toxin to a different site in the Na^+ channel (27). The properties of BTX have suggested that it reacts preferentially with the open form of the Na^+ channel. It also seems likely that various local anesthetics antagonize interaction or binding of BTX to the Na^+ channels (see refs. 28–30). Indeed, there is considerable evidence that local anesthetics and other compounds with local anesthetic properties reduce the action of BTX by inhibiting the binding of BTX in a mutually exclusive fashion. The converse may also be true. Procaine and lidocaine inhibit BTX-induced depolarization in skeletal muscle (13), and procaine, trimecaine, and QX-572 inhibit BTX effects in voltage-clamped nerve fibers of frog (3, 14). Conversely, BTX appears to reduce potency of local anesthetics in nerve fibers (14, 15). Kinetic studies achieved by measuring the influx of $^{22}Na^+$ into neuroblastoma cells following activation of Na^+ channels by BTX have shown that benzocaine and QX-572 (31), yohimbine (32), lidocaine, procainamide, and diphenylhydantoin (28) inhibit BTX-induced depolarization competitively. Similarly, employing the change in the equilibrium distribution of [³H]$TPMP^+$ as an indirect measure of BTX activation of Na^+ channels, our results indicate that a selection of local anesthetics with diverse structures inhibit the action of BTX in a competitive manner. Thus it seems clear that compounds with a wide range of structures that bear little apparent similarity to the structure of BTX give rise to competitive inhibition patterns with BTX-dependent changes in membrane potential or ion flux.

The demonstration that [³H]BTX-B interacts selectively with the BTX-binding site on Na^+ channels (5, 6) provided the basis for a direct measurement of the effects of local anesthetics on binding of the toxin to this site. There was an excellent correlation between the ability of seven local anesthetics to inhibit binding of [³H]BTX-B and their ability to inhibit BTX-induced depolarization in guinea pig vesicular preparations. The sole exception of the compounds tested was lidocaine ethiodide. Perhaps the enhanced affinity of this lipid-insoluble quaternary amine for the scorpion toxin-activated BTX-binding site as compared with its lesser ability to inhibit BTX-induced depolarization is related to a greatly enhanced accessibility to the binding site in the presence of scorpion toxin when most of the BTX-sensitive channels are in the open form. This difference may be especially large in the case of lidocaine ethiodide, which is essentially inactive against Na^+ currents when applied externally and an active blocker when applied internally to intact nerve preparations (11, 24). For the remaining six local anesthetics, the inhibition constants obtained under the two assay conditions were remarkably close. The ratios

of the values versus BTX-induced depolarization to those versus binding of [^{3}H]BTX-B ranged from 1.2 to 3.7. The inhibition constant versus [^{3}H]BTX-B binding was generally the lower value. It should be emphasized that, although local anesthetics are mutually exclusive (competitive) inhibitors of BTX-induced depolarization and directly compete with binding of [^{3}H]BTX-B, this does not necessarily indicate that these measures are directly related or that the mechanism of action is a direct steric interaction at a single, common binding site. On the contrary, the structural dissimilarity between BTX and the competing species, the ability of the Na^+ channel to undergo allosteric activation by BTX and other toxins (27), and the demonstration that certain local anesthetics yield noncompetitive inhibition patterns against a partial agonist of the Na^+ channel (28) suggest that local anesthetics may act either through an indirect allosteric inhibition or through multiple binding sites, one of which is common with a BTX-binding site. It should be noted that in nerve fibers pretreated with BTX, local anesthetics do not appear able to displace BTX (14, 15). Under such conditions the local anesthetics are not particularly effective in blocking Na^+ conductances. Measurements of the inhibition of [^{3}H]BTX-B binding appears to provide a rapid and reliable estimate of local anesthetic potency of both classical local anesthetics and a variety of other compounds and thus provides a useful probe for investigating both the mechanism of action of local anesthetics and for detailed structure-activity correlations for local anesthetic sites on the Na^+ channel.

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