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INTERACTION OF YOHIMBINE WITH BATRACHOTOXININ BINDING TO MOUSE BRAIN SODIUM CHANNELS

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Abstract—To study the local anesthetic properties of yohimbine in more detail, its effect was examined *in vitro* on the scorpion toxin-enhanced specific binding of [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B) to the gating complex in sodium channel preparations from mouse brain cortex. Both equilibrium and kinetic experiments were carried out. Yohimbine inhibited the specific binding of [^{3}H]BTX-B in the vesicular preparation with an IC_{50} value of 2.2×10^{-5} M. This is about one order of magnitude higher than the concentration required for antagonism via the alpha$_2$-adrenoceptors; however, yohimbine is 7-fold more potent in inhibiting [^{3}H]BTX-B binding than lidocaine. In a concentration-dependent manner, yohimbine increased the dissociation constant (K_d) of high-affinity [^{3}H]BTX-B binding without changing the maximal binding capacity (B_{max}). The dissociation rate constant was not affected by yohimbine, suggesting competitive inhibition as opposed to the action of local anesthetics involving an allosteric action via receptor sites distinct from the BTX site. Alpha$_2$-adrenoceptors are apparently not involved because clonidine and α-methyl-noradrenaline had no appreciable effect on [^{3}H]BTX-B binding and did not antagonize the inhibitory effect of yohimbine. The present findings indicate a mechanism of local anesthetic action of yohimbine that differs from that of other local anesthetics such as tetracaine and lidocaine involving direct binding to the BTX site, thereby stabilizing a non-permeable form of the sodium channel.

Local anesthetics, such as procaine and tertiary and quaternary derivatives of lidocaine, are known to block the sodium current [1]. Electrophysiological and biochemical evidence suggests that the voltage-dependent sodium channel is the site of local anesthetic action. For instance, these drugs inhibit the veratridine-stimulated Na^+ uptake into rat brain homogenates [2] and the binding of [^{3}H]BTX-B‡ to sodium channels of rat or mouse brain [3–5], but they do not affect the binding of [^{3}H]TTX or [^{125}I]ScTX [5, 6], to site 1 and site 3, respectively, on the sodium channel [7]. The action of local anesthetics on [^{3}H]BTX-B binding involves a specific receptor, and although their inhibitory effect appears to be competitive, the mechanism is best described by the allosteric model for heterotropic cooperative interactions [5, 8].

Yohimbine is an indolalkylamine alkaloid that is obtained from *Coryanthe yohimbe*, a species of the family *Apocynaseae*. Yohimbine has many effects on the central and peripheral nervous systems. It is an alpha$_2$-adrenoceptor antagonist [9], it has a central effect on the cardiovascular system [10] and on the intermediary metabolism of tryptophan [11], and it blocks the peripheral 5-hydroxytryptamine receptors [11]. Yohimbine is well known to have local anesthetic activity; it is 3-fold more potent than procaine and 7-fold more potent than lidocaine in inhibiting [^{3}H]BTX-B binding [3, 12]. Lipicky *et al.* [13] demonstrated that yohimbine inhibits the sodium current by acting on the channel gating mechanism rather than by occluding the pore. In their model they assume that yohimbine binds to the gating molecules of sodium channels and modifies their opening and closing rates. Huang *et al.* [12] showed that yohimbine reduces BTX-induced permeability to sodium in mouse neuroblastoma cells, supporting the conclusion of Lipicky *et al.* [13].

The effect of yohimbine on sodium current was investigated in frog Ranvier node membranes by Revenko *et al.* [14], who found that the drug decreases the outward sodium current much more strongly than the inward current, and interacts mainly with open sodium channels. They suggested that yohimbine and local anesthetics share a common receptor in sodium channels. A study of the binding of [^{3}H]yohimbine to alpha$_2$-adrenoceptors in rat brain synaptosomes showed that veratridine, a lipid-soluble neurotoxin, competes with [^{3}H]yohimbine for its binding to alpha$_2$-adrenoceptors, indicating some pharmacological relationship between sodium channels and alpha$_2$-adrenoceptors [15].

In the present study, [^{3}H]BTX-B binding to mouse brain cortex membranes was used as a tool to investigate the interactions of yohimbine with the gating

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‡ Abbreviations: BTX-B, batrachotoxinin A 20-α-benzoate; TTX, tetrodotoxin; ScTX, scorpion toxin; HEPES, *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid; K_d, equilibrium dissociation constant; B_{max}, maximal binding capacity; IC_{50}, concentration required to inhibit binding by 50%; and k_{-1}, dissociation rate constant.

mechanism of voltage-dependent sodium channels. The present work was undertaken to compare the effects of yohimbine on the [^{3}H]BTX-B binding with those of other local anesthetics described previously; to assess whether yohimbine is a competitive inhibitor of BTX-B binding with or without allosteric modulation; and finally to evaluate whether there is a pharmacological similarity between BTX receptors on voltage-sensitive sodium channels and alpha$_2$-adrenoceptors.

MATERIALS AND METHODS

Animals. For all our experiments we used female BALB/cBy mice, 8–12 weeks of age, weighing 21–24 g, from the breeding colony on Ward's Island. The animals were kept on a 12-hr light/dark cycle (7:00 a.m./7:00 p.m. light), with food and water available *ad lib.*

Chemicals. [*Benzoyl*-2,5-^{3}H]Batrachotoxinin A 20-α-benzoate with a specific activity of 50 Ci/mmol was obtained from New England Nuclear (Boston, MA, U.S.A.). Bovine serum albumin, choline-chloride, *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), α-D(+)-glucose, scorpion venom *(Leiurus quinquestriatus)* toxin (ScTX), tetrodotoxin (TTX), and aconitine were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tris-(hydroxymethyl)-aminomethane, potassium chloride, calcium chloride, and magnesium sulfate were from the Fischer Scientific Co. (Springfield, NJ, U.S.A.). Batrachotoxin (BTX) was supplied by Dr. J. W. Daly (NIH, Bethesda, MD, U.S.A.).

[^{3}H]*BTX-B binding assays.* Preparations enriched in vesicular elements were obtained from mouse brain cortex by the procedure of Creveling *et al.* [3]. The major components of this preparation appear to be resealed postsynaptic elements and attached presynaptic endings. Following decapitation of the mice, the brains were removed rapidly and placed immediately in ice-cold 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 (400 mg) was homogenized by hand (10–12 strokes) in 1 ml of buffer and centrifuged at 1000 *g* for 15 min. The supernatant fraction was discarded, and the pellet was resuspended in 5 ml of fresh buffer for assay.

Specific high-affinity binding at 5 nM [^{3}H]BTX-B was measured by a modification of the technique described by Reith *et al.* [8] and Catterall *et al.* [16]. The vesicular preparation was placed in standard incubation medium containing 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM HEPES buffer and 130 mM choline-Cl for the maintenance of the membrane potential in the absence of sodium ions (adjusted to pH 7.4 with 500 mM Tris). The incubation was carried out in a total volume of 550 μl containing 1.25 μM TTX, 1.25 mg/ml bovine serum albumin, 25 μg/ml ScTX, and 400 μg protein of the vesicular preparation, at 36° for 120 min. TTX was included, because it effectively blocks the influx of sodium ions, and at 36° has no effect on [^{3}H]BTX-B binding [17]. ScTX was present because it enhances the apparent affinity of the [^{3}H]BTX-B to its binding sites by an allosteric mechanism [16]. The assays were terminated by addition of 5 ml of ice-cold wash medium, consisting of 163 mM choline-Cl, 5 mM HEPES buffer, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 1 mg/ml bovine serum albumin (adjusted to pH 7.4 with 500 mM Tris), and filtration through Whatman GF/C glass-fiber filters with a cell harvester (Brandel); two 5-ml washes were used to rinse the filters. Radioactivity was measured by scintillation spectroscopy with an efficiency of approximately 50%. Protein concentrations were determined by the method of Lowry *et al.* [18] after removal of the HEPES buffer by precipitating protein with 1% (v/v) perchloric acid, centrifuging at 30,000 *g*, and dissolving the pellet in 0.5 M NaOH.

Nonspecific binding of [^{3}H]BTX-B was defined with a final concentration of 0.2 mM aconitine added as concentrated stock in ethanol; the final ethanol concentration was not more than 1% (v/v) and had little or no effect on total binding. The average value for nonspecific binding with aconitine amounted to approximately 22% of the total binding of 5 nM [^{3}H]BTX-B. Filter binding of [^{3}H]BTX-B in the absence of vesicular preparation was negligible. In experiments aimed at measuring the dissociation rate of [^{3}H]BTX-B, the dissociation was initiated with a final concentration of 0.2 mM aconitine and various concentrations of yohimbine by addition of the compounds in 30 μl of a stock solution as indicated.

Data analysis. Six different concentrations of yohimbine (three below and three above IC_{50}) were used to assess its potency. The IC_{50} value was computed with the ALLFIT program developed by DeLean *et al.* [19]. Equilibrium binding data from saturation analysis with various concentrations of BTX were fitted to a one-site model, and the dissociation constant (K_d) and maximal number of binding sites (B_{max}) were determined by nonlinear regression analysis with the LIGAND program [20]. The same Hill coefficients were obtained whether vesicular preparations were incubated for 60 or 120 min with [^{3}H]BTX-B; after 60 min of incubation the total and specific binding had reached a plateau. Dissociation rate constants, k_{-1}, were calculated by least-squares linear regression analysis for $\ln(SB/SB_0) = -k_{-1}t$, in which SB is specific binding at time t and SB_0 is specific binding at time zero.

One-way ANOVA was used to test the existence of differences between the means of the drugs and of their corresponding controls.

RESULTS

Binding of [^{3}H]*batrachotoxinin A 20-α-benzoate.* The effect of ScTX on the binding of 5 nM [^{3}H]BTX-B to vesicular preparations of mouse brain cortex is illustrated in Fig. 1. ScTX enhanced the specific binding of [^{3}H]BTX-B in a concentration range from 1.0 to 100 μg/ml by 3.6-fold in agreement with results reported by Catterall *et al.* [16]. A concentration of 8 μg/ml of ScTX was required for a half-maximal increase in [^{3}H]BTX-B binding. In subsequent experiments, 25 μg/ml of ScTX was used. The Scatchard plots of the BTX binding were linear, indicating a single class of receptor sites for [^{3}H]BTX-B with a K_d of 27 nM and a B_{max} of 3.54 pmol/mg protein in agreement with the previous results of

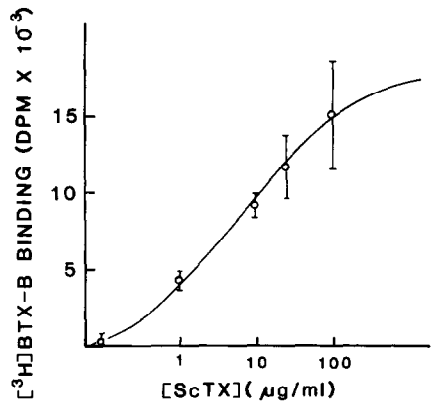

Fig. 1. Enhancement of specific [^{3}H]BTX-B binding by scorpion toxin. [^{3}H]BTX-B binding was measured as described under Materials and Methods in a volume of 550 μl in the presence of 1.25 μM TTX, 5 nM [^{3}H]BTX-B, and increasing concentrations of ScTX. Nonspecific binding was measured in the presence of 0.2 mM aconitine and did not differ in the presence or absence of 100 μg/ml of ScTX.

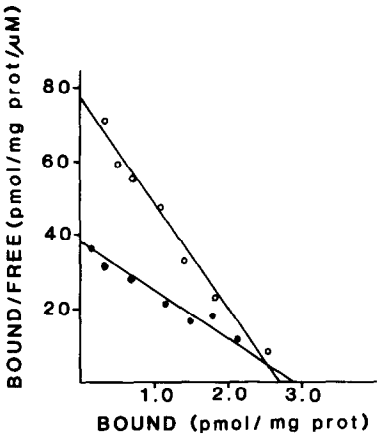

Fig. 3. Scatchard analysis of the effect of yohimbine on binding of [^{3}H]BTX-B. Binding of 5 nM [^{3}H]BTX-B was measured in a total volume of 550 μl of incubation mixture in the presence of 1.25 μM TTX, 25 μg/ml ScTX, and increasing concentrations of unlabeled BTX (5, 10, 20, 50, 100, and 200 nM). Key (○) control; (●) 10^{-5} M yohimbine. Points shown are those obtained from a single experiment, performed in triplicate, which was replicated twice. Data were analyzed with the LIGAND computer program. The lines are drawn for B_{max} values of 2.5 and 3.0 pmol/mg protein and for K_d values of 32.6 and 83.4 nM in control and in the presence of 10^{-5} M yohimbine respectively.

Creveling *et al.* [3]. In the presence of 8 μg/ml ScTX, the analysis of the [^{3}H]BTX-B binding with increasing concentrations of unlabeled BTX resulted in upward-concave Scatchard curves, indicating the presence of more binding sites, and a more complex system for [^{3}H]BTX-B (data not shown).

Effects of yohimbine on [^{3}H]*BTX-B binding.* Yohimbine was found to inhibit specific binding of [^{3}H]BTX-B to mouse brain cortex vesicular preparations, without affecting the nonspecific binding. The following equilibrium and kinetic results are in agreement with yohimbine being a competitive inhibitor competing with BTX for the BTX receptor sites.

Yohimbine inhibited the binding of [^{3}H]BTX-B with an IC_{50} of 2.2×10^{-5} M (Fig. 2), which corresponds to the results of McNeal *et al.* [4]. To assess whether yohimbine interacts with [^{3}H]BTX-B receptors in the same manner as other local anesthetic drugs [3–5, 8*], the mechanism of the inhibition of [^{3}H]BTX-B binding by yohimbine was investigated in both equilibrium and dissociation experiments.

Three different concentrations of yohimbine were analyzed with six or seven concentrations of unlabeled BTX in equilibrium experiments (Fig. 3 and Table 1). Yohimbine increased the K_d of the BTX-

* C. R. Creveling, G. A. Lewandowsky and J. W. Daly, Sixteenth Annual Meeting of the Society for Neuroscience, Abstr. 410.6 (1986).

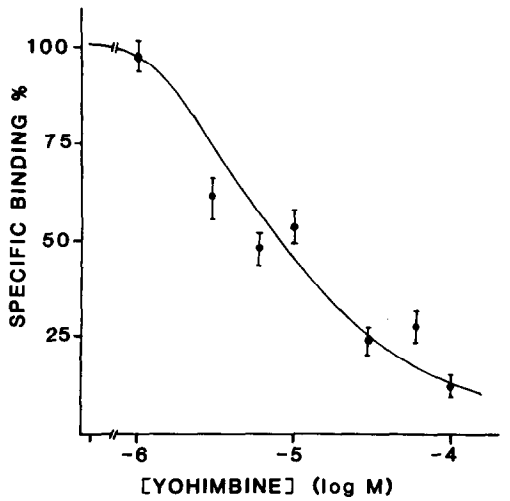

Fig. 2. Dose–response curve for yohimbine inhibition of [^{3}H]BTX-B binding. The [^{3}H]BTX-B binding was measured as described in Materials and Methods in 550 μl of incubation medium in the presence of 1.25 μM TTX and 25 μg/ml ScTX. The nonspecific binding in the presence of 0.2 mM aconitine was subtracted from the data. Points shown are those obtained in two experiments, performed in triplicate. The mean values ± SEM are presented.

Table 1. Effect of yohimbine on the dissociation constant and the maximal binding capacity of [^{3}H]BTX-B

Concentration of yohimbine (M)	K_d (nM)	B_{max} (pmol/mg protein)
Control	32.6 ± 6.3	2.54 ± 0.31
5×10^{-6}	80.4 ± 12.2	3.01 ± 0.34
10^{-5}	83.4 ± 11.9	2.94 ± 0.32
5×10^{-5}	107.3 ± 2.1	1.91 ± 0.30

The binding of BTX was measured as described in Materials and Methods. Values are the mean of three experiments ± SE. K_d values are significantly different, $P < 0.01$ (one-way ANOVA).

B binding in a concentration-dependent manner, without affecting the B_{max}, suggesting that yohimbine competes with the [^{3}H]BTX-B for its binding sites. The Hill coefficients of cocaine congeners are close to unity [8] and the Hill coefficient for yohimbine is 0.84 ± 0.10 (N = 3), suggesting that these drugs are competitive inhibitors interacting with a single class of binding sites for [^{3}H]BTX-B. Kinetic studies of the effects of local anesthetics on the dissociation rate of the BTX–receptor complex indicated an indirect allosteric competitive inhibition of binding of the [^{3}H]BTX-B that increased the dissociation rate of BTX and shifted the sodium channels to an inactive form [5, 8]. The local anesthetic drug tetracaine at a concentration of 5 μM enhanced the rate of dissociation under the present conditions from 0.0072 to 0.0120 min^{-1} (data not shown). To determine whether yohimbine has a similar effect, the aconitine-induced dissociation of the [^{3}H]BTX-B was measured in the absence and the presence of three different concentrations of yohimbine. Yohimbine had no effect on the rate of the aconitine-induced dissociation of [^{3}H]BTX-B (Fig. 4). The values of the rate constants (k_{-1}) in the absence and in the presence of 5×10^{-6}, 10^{-5}, and 10^{-4} M yohimbine ranged from 0.0066 to 0.0074 min^{-1}. These findings strengthen the idea that yohimbine is a competitive inhibitor of [^{3}H]BTX-B binding and that its effect is different from the effects of other local anesthetics.

Effect of alpha$_2$-adrenoceptor agonists on [^{3}H]BTX-B binding. To assess whether the effects of yohimbine are due to its alpha$_2$-adrenoceptor antagonistic action, the effects of two alpha$_2$-adrenoceptor agonists were tested on the [^{3}H]BTX-B binding alone and in the presence of yohimbine. Clonidine and α-methyl-noradrenaline did not have much effect on [^{3}H]BTX-B binding at concentrations up to 2×10^{-3} M. These compounds did not antagonize the effect of yohimbine even at concentrations of 2×10^{-5} and 5×10^{-4} M, which are two orders of magnitude higher than the concentrations commonly used in pharmacological experiments involving alpha$_2$-adrenoceptors (data not shown).

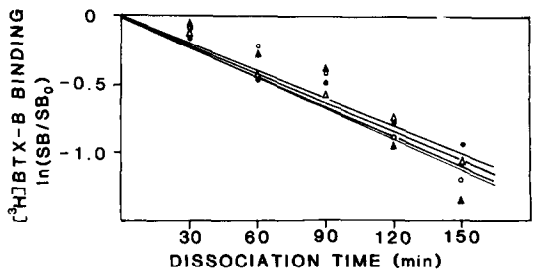

Fig. 4. Time course of the dissociation of [^{3}H]BTX-B induced by aconitine in the absence and in the presence of yohimbine. Binding of 5 nM [^{3}H]BTX-B was measured in the presence of 1.25 μM TTX and 25 μg/ml ScTX in 550 μl of incubation medium. Dissociation of [^{3}H]BTX-B was measured as described in Materials and Methods. Specific binding (SB) at zero time (SB_0) was, on the average, 252 fmol/mg protein. Nonspecific binding did not change with time. Key: (●) no yohimbine; (○) 5×10^{-6} M yohimbine; (△) 10^{-5} M yohimbine; and (▲) 10^{-4} M yohimbine. The k_{-1} values were 0.0074, 0.0067, 0.0066, and 0.0072 respectively. Data shown are from a single experiment assayed in triplicate, which was replicated once.

DISCUSSION

Receptors for neurotoxins on sodium channels have been classified by Catterall [21] into three major classes. Two of these, the TTX site and the ScTX site, do not appear to be influenced by local anesthetics [5] or by yohimbine [6]. In contrast, these compounds do affect the third site, the BTX binding site, which is associated with the gating mechanism of the channel [3, 5, 16]. The binding of BTX to the sodium channel prevents inactivation of the channel resulting in a massive influx of sodium ions and a persistent membrane depolarization [22]. ScTX increases the fraction of sodium channels activated by BTX and shifts the concentration–effect curves for BTX to lower concentrations [23]. These effects are quantitatively described by an allosteric model with heterotropic cooperativity [21]. ScTX appears to reduce the energy required for activation of sodium channels by the lipid-soluble toxins and increases the fraction of the channels in the conducting stage with high affinity for BTX [7]. In the present experiments with mouse brain vesicular preparations, ScTX enhanced the binding of [^{3}H]BTX-B submaximally at 8 μg/ml (Fig. 1).

The present results show an apparent competitive mechanism of inhibition for the effect of yohimbine on the binding of [^{3}H]BTX-B (Fig. 3, Table 1). This result is identical to that for the inhibition by local anesthetic drugs of [^{3}H]BTX-B binding [5]. Recent studies suggest that the binding of local anesthetics to sites distinct from those for [^{3}H]BTX-B promotes an allosteric inhibition of binding of the [^{3}H]BTX-B, thus increasing the dissociation rate of BTX and shifting the sodium channel to an inactive form [5, 8]. In our biochemical experiments we can distinguish only the conducting and non-conducting state of the channel. In contrast, in electrophysiological experiments there are at least two non-conducting states (the resting state at negative resting potentials and the inactivated state after maintained depolarization) and one conducting state (the transient open state existing for only a few milliseconds during a depolarization). It is still under debate whether local anesthetics bind more tightly to the open state than to the resting state or whether they bind more tightly to the inactivated state than to the resting state [5, 24, 25]. Regardless, the biochemical approach can detect allosteric interactions between sites for inhibitors and sites for BTX by measuring the rate of dissociation of [^{3}H]BTX-B binding [5, 8]. The present results (Fig. 4) clearly show that yohimbine differs from other local anesthetic drugs in the mechanism by which it affects [^{3}H]BTX-B binding. Yohimbine had no effect on the aconitine-induced dissociation of [^{3}H]BTX-B binding. In contrast, the local anesthetic drug tetracaine enhanced the rate of dissociation under the present conditions (data not shown). The most parsimonious explanation for the present results from the equilibrium and dissociation experiments is that yohimbine is a competitive inhibitor that binds to the same receptor sites as [^{3}H]BTX-B.

Such a mechanism is consonant with previous results from electrophysiological and biochemical experiments on the local anesthetic effects of yohimbine. For instance, Lipicky *et al.* [13] showed that yohimbine has tonic and "use-dependent" effects on sodium current, which is completely reversible. The effect on sodium current can be described by a decrease in the amplitude. Furthermore, Huang *et al.* [12] demonstrated a competitive mechanism for the inhibition by yohimbine of BTX-stimulated sodium uptake into neuroblastoma cells. Finally, yohimbine competitively inhibits the efflux of $^{86}Rb^+$ through activated sodium channels in brain synaptosomal preparations [6]. Thus, yohimbine probably interacts with the gating proteins, either by an allosteric modulation or by an action at the same site that binds BTX. The results presented in this study give support to the latter possibility. In addition, the present results make it less attractive to consider the possibility that yohimbine blocks sodium channel activity by occluding the pore. Other possible effects involving different receptor sites are unlikely as it was demonstrated by Frelin *et al.* [6] that yohimbine is without effect on labeled ScTX or TTX binding. Thus, most likely, yohimbine stabilizes a non-permeable form of the channel by binding to the BTX site. The possibility exists that this site represents the low-affinity component of [^{3}H]yohimbine binding observed in rat cerebral cortex [26], whereas the high-affinity [^{3}H]yohimbine binding represents the classical alpha$_2$-adrenoceptor. This could perhaps contribute to the observed similarities between the potencies of alpha$_2$-antagonists in inhibiting [^{3}H]yohimbine binding and their potencies in interacting with the efflux of $^{86}Rb^+$ through synaptosomal sodium channels [6]. The lack of activity of alpha$_2$-adrenoceptor agonists in the above study is consonant with the present finding that clonidine and α-methyl-noradrenaline did not affect [^{3}H]BTX-B binding. In addition, these agents did not antagonize the inhibitory effect of yohimbine, excluding the direct involvement of alpha$_2$-adrenoceptors in this effect.

The effect of yohimbine shown here suggests that local anesthetic action of compounds can be achieved by mechanisms other than those involving receptors in the channel distinct from the BTX site. This conclusion is consonant with the previous suggestion [1] that there are multiple sites for local anesthetics in the sodium channels. It is conceivable that this is clinically useful by administering a mixture of local anesthetics that bind to different receptors.

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