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Batrachotoxin changes the properties of the muscarinic receptor in rat brain and heart: Possible interaction(s) between muscarinic receptors and sodium channels

(local anesthetic/muscarinic agonist/different mode of binding/receptor cross-talk/veratridine)

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ABSTRACT **The effects of Na^+-channel activator batrachotoxin (BTX) on the binding properties of muscarinic receptors in homogenates of rat brain and heart were studied. BTX enhanced the affinity for the binding of the agonists carbamoylcholine and acetylcholine to the muscarinic receptors in brainstem and ventricle, but not in the cerebral cortex. Analysis of the data according to a two-site model for agonist binding indicated that the effect of BTX was to increase the affinity of the agonists to the high-affinity site. Guanyl nucleotides, known to induce interconversion of high-affinity agonist binding sites to the low-affinity state, canceled the effect of BTX on carbamoylcholine and acetylcholine binding. BTX had no effect on the binding of the agonist oxotremorine or on the binding of the antagonist [^{3}H]-*N*-methyl-4-piperidyl benzilate. The local anesthetics dibucaine and tetracaine antagonized the effect of BTX on the binding of muscarinic agonists at concentrations known to inhibit the activation of Na^+ channels by BTX. On the basis of these findings, we propose that in specific tissues the muscarinic receptors may interact with the BTX binding site (Na^+ channels).**

Previous studies on ligand binding to the muscarinic receptors demonstrated that the binding of muscarinic antagonists from the benzilate or tropate classes yields binding iotherms that fit a single dissociation constant (reviewed in ref. 1). On the other hand, the binding of agonists indicates the existence of a heterogenous population of binding sites that differ in their affinities to agonists (for review, see refs. 1 and 2). Thus, it appears that the binding of muscarinic agonists is more sensitive to the state of the receptor, a situation that makes agonist binding a better tool for detecting alterations in the state of the muscarinic binding sites.

Recent studies using antiarrhythmic and local anesthetic drugs (3–7) provided evidence for a complex mode of interaction between these drugs and the muscarinic receptors. A recent study (7) raised the possibility that the site through which these drugs affect the muscarinic receptor is analogous to the voltage-sensitive sodium channel. Preliminary experiments (7) on the effects of batrachotoxin (BTX) on the muscarinic system lend further support to this hypothesis. BTX interacts specifically with sites on the sodium channel (8); therefore, studies on the effects of this drug on the muscarinic sites can reveal interactions between Na^+ channels and the muscarinic receptors.

Voltage-sensitive sodium channels in nerve and neuroblastoma cells have three separate receptor sites for neurotoxins (reviewed in refs. 9–12). BTX and several other alkaloids (e.g., veratridine and aconitine) interact with one site and induce persistent activation of Na^+ channels. Polypeptide toxins (e.g., scorpion toxin) bind to another site that interacts allosterically with the BTX site. Other inhibitors, such as tetrodotoxin, bind to a third site and inhibit ion flux through the Na^+ channel.

The objective of the work described here was to gain further insight into the possible association between muscarinic receptors and sodium channels. For this purpose, we have examined the effect induced by BTX on the binding of muscarinic ligands in homogenates prepared from rat brain and heart.

MATERIALS AND METHODS

Materials. [^{3}H]-*N*-methyl-4-piperidyl benzilate ([^{3}H]-4NMPB; 69.7 Ci/mmol; 1 Ci = 37 GBq) was the same preparation described and used previously (6, 7).

BTX, aconitine, veratridine, dibucaine, and tetracaine were kindly supplied by J. Daly (Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health). BTX was dissolved in ethanol at 500 times the concentration used in the experiments and was added to the assay mixture immediately before use. The storage period did not exceed 3 weeks.

Methods. *Tissue preparation.* Adult male rats of the CD strain were obtained from Levinstein's Farm (Yokneam, Israel) and maintained in an air conditioned room at 24 ± 2°C, under a cycle of 14 hr (0500–1900) of light (fluorescent illumination) and 10 hr of darkness. Food from Assia Maabarot (Tel Aviv) and water were supplied ad lib. Rats aged 3–4 months and weighing 190–250 g were decapitated (between 1000 and noon) and their brains or hearts were rapidly removed. The brainstem and the cortex were dissected out in a cold room. The tissues were homogenized within 15 min and were used immediately in the binding experiments.

Binding assay. Heart ventricles were cut up finely with scissors, mixed with 9 vol of modified Krebs–Henseleit solution (25 mM Tris·HCl/118 mM NaCl/4.7 mM KCl/1.2 mM $CaCl_2$/0.5 mM $MgCl_2$/1.0 mM NaH_2PO_4/11.1 mM glucose, pH 7.4) and homogenized at setting 7 on an Ultra-Turrax (Ika-Werk Instruments, Cincinnati, OH) with three 15-sec bursts separated by 30-sec pauses. The homogenates were filtered through three layers of cheesecloth and centrifuged twice in the same buffer solution (17,000 rpm; 15 min). Brain regions were homogenized as described in detail (13) to yield a 4% homogenate (wt/vol). Homogenates prepared from the brainstem (two rats), the cortex (one rat), and the ventricle (three rats) were used for binding assays as follows (6, 13): the tissue preparation was incubated at 36°C with

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Abbreviations: 4NMPB, *N*-methyl-4-piperidyl benzilate; BTX, batrachotoxin; p[NH]ppG, guanylyl imidodiphosphate; IC_{50}, concentration that inhibits response by 50%.

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BTX alone or together with other Na^+-channel drugs or local anesthetics in modified Krebs–Henseleit solution, followed by incubation (36°C, 30 min) of 50 μl of homogenate in 190 μl of buffer solution containing various amounts of [^{3}H]-4NMPB, a radiolabeled muscarinic antagonist.

The effect of BTX and the other drugs on the binding of muscarinic agonists was investigated by means of competition experiments using unlabeled agonist and [^{3}H]4NMPB (6, 14). In the case of acetylcholine, the binding studies were performed in the presence of 200 μM diisopropyl fluorophosphate (15). Tissue preparations were incubated in the same buffer in a series of test tubes containing different agonist concentrations, 2 nM [^{3}H]4NMPB, and a fixed concentration of the toxin and/or additional drug. This [^{3}H]4NMPB concentration was chosen so as to produce high occupancy of the sites (≈80%) with low nonspecific binding.

After incubation for 30 min at 36°C, ice-cold Krebs–Henseleit solution (3 ml) was added and the contents were passed rapidly by suction through a glass filter (Whatman GF/C; 25 mm diameter). The filters were washed three times in 3 ml of the same solution. The radioactivity was measured by liquid scintillation spectrometry using a Packard Tricarb 300 (6, 13). All procedures were completed within <10 sec. Binding assays were performed in triplicate, together with triplicate control samples containing 5 μM unlabeled atropine. Specific binding was defined as the total binding minus the nonspecific binding in the presence of atropine. Protein was determined by the method of Lowry, using bovine serum albumin as a standard.

Data analysis. Agonist binding data obtained from competition experiments were computer analyzed using a nonlinear regression curve-fitting procedure (program DMDPAR, Nov. 1978 revision, developed at the Health Science Computing Facility, University of California, Los Angeles, CA) as described (14). The data were fitted to a model assuming two populations of noninteracting binding sites with high and low affinities toward agonists, but with similar affinity to the antagonist [^{3}H]4NMPB.

RESULTS

Effects of BTX on the Binding of Muscarinic Ligands. The binding of [^{3}H]4NMPB to brainstem, cortex, and ventricle preparations was similar in the presence or absence of 1 μM BTX. On the other hand, the neurotoxin affected the displacement of [^{3}H]4NMPB by the muscarinic agonists carbamoylcholine and acetylcholine in homogenates from brainstem and ventricle, inducing a leftward shift in the displacement curves (Fig. 1). A similar effect (enhanced agonist affinity) was also observed in direct binding studies using [^{3}H]acetylcholine. No such effects were observed in cortex homogenates. Unlike the situation with carbamoylcholine and acetylcholine, no effects of BTX could be observed in any of the above homogenates with oxotremorine (data not shown).

Analysis of the data according to a two-site model for agonist binding indicated that BTX did not change the relative proportions of high- and low-affinity agonist binding sites, but rather that it increased the affinity of the agonist toward the high-affinity site, with insignificant effects on the binding to the low-affinity sites. This phenomenon is shown in Table 1 (lines 1, 2) for carbamoylcholine binding to brainstem homogenates. Competition experiments were then carried out using brainstem preparations, carbamoylcholine and acetylcholine as the agonists, and several concentrations of BTX. The dose–response curves thus obtained for the changes induced by BTX on the high-affinity sites are depicted in Fig. 2, demonstrating very strong dependence on the neurotoxin concentration, with a maximal effect at ≈1 μM BTX. It should be noted that experiments on the effect of BTX on the binding of the above two muscarinic agonists yielded similar results whether conducted in the presence or in the absence of Na^+ ions, indicating that if closed structures (e.g., synaptosomes) are present, it is very unlikely that the observed effects of BTX on the muscarinic receptors are due to depolarization of the membranes or sodium fluxes.

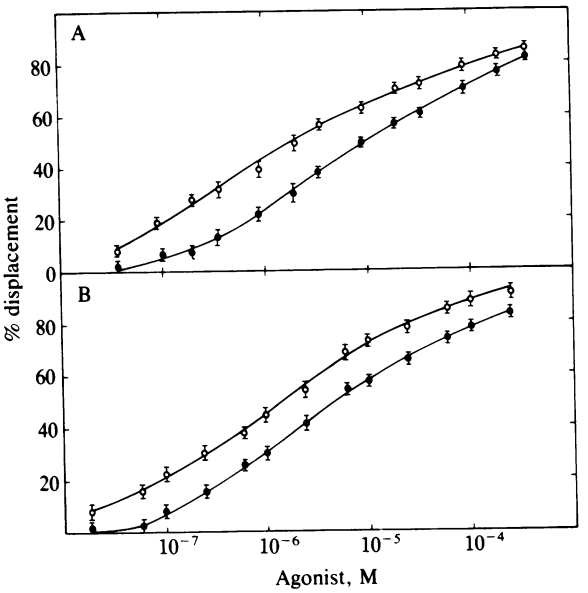

FIG. 1. Inhibition curves of the binding of 2 nM [^{3}H]4NMPB by carbamoylcholine (*A*) and acetylcholine (*B*) in the brainstem in the absence (●) and presence (○) of 1 μM BTX. [^{3}H]4NMPB binding site concentration was 0.12 nM. Values shown are means ± SEM of five experiments. Each experiment was performed in triplicate.

Effect of Guanylyl Imidodiphosphate (p[NH]ppG) on the Interaction of BTX with Muscarinic Binding Sites. BTX affects the high-affinity state in the brainstem preparation, which is enriched with high-affinity sites. On the other hand, it has no effect in the cortex, which is enriched with low-affinity sites (1, 2). Guanyl nucleotides are known to induce interconversion of high-affinity agonist binding sites to the low-affinity state (reviewed in ref. 1). It was therefore of interest to investigate their effects on the interaction of BTX with the muscarinic sites in the brainstem. As shown in Table 1 (compare line 1 with lines 3 and 4), the effect of p[NH]ppG treatment in the presence of BTX produced the same results

Table 1. Effect of p[NH]ppG on the displacement of 2 nM [^{3}H]4NMPB by carbamoylcholine in the presence of BTX

	α	K_H, M	K_L, M	$K_H/K_H^{control}$
Control	56.9 ± 3.3	$5.1 \pm 0.9 \times 10^{-7}$	$6.3 \pm 1.4 \times 10^{-5}$	1
With 1 μM BTX	56.4 ± 3.1	$6.7 \pm 1.4 \times 10^{-8}$	$4.2 \pm 0.8 \times 10^{-5}$	0.13
With 1 μM BTX/200 μM p[NH]ppG	43.0 ± 3.5	$6.3 \pm 1.3 \times 10^{-7}$	$6.0 \pm 1.2 \times 10^{-5}$	1.2
With 200 μM p[NH]ppG	40.5 ± 2.5	$6.1 \pm 0.3 \times 10^{-7}$	$5.1 \pm 1.1 \times 10^{-5}$	1.2

Binding and computer analysis of carbamoylcholine binding to brainstem membranes were performed as described. The values depicted are means ± SEM of five experiments. α is the fraction of high affinity agonist binding to sites. K_H and K_L are the dissociation constants for agonist binding to the high- and low-affinity sites, respectively.

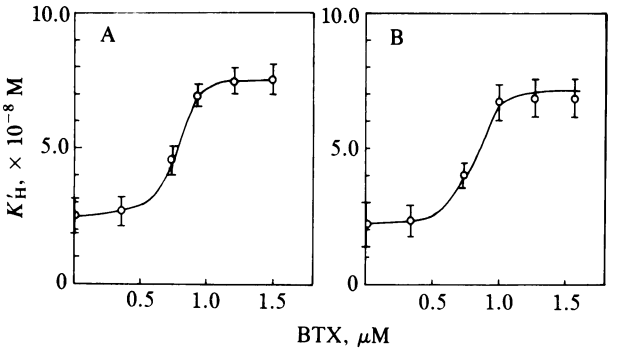

FIG. 2. Dose dependence of the perturbation induced by BTX on the association constants (K'_H) for the binding of carbamoylcholine (*A*) and acetylcholine (*B*) to the high-affinity sites. The data shown in Fig. 1 were analyzed for a two-site model. The K'_H values obtained by the computer analysis were plotted as a function of BTX concentration.

as those observed in the absence of BTX—namely, it induced interconversion of high-affinity to low-affinity sites (α, the fraction of high-affinity sites, is 57% in the control as compared to 40% after p[NH]ppG treatment). This effect of p[NH]ppG was accompanied by a cancelation of the effect induced by BTX on the high-affinity binding sites, whose affinity toward agonists returned to the control value (Table 1, compare lines 2 and 3).

Effect of Alkaloids on the Interaction of BTX with the Muscarinic Receptors. Certain other alkaloids, such as aconitine and veratridine, are known to interact with the same site on the Na^+ channel as BTX, although they are much less potent and efficacious (see refs. 12 and 16 and references therein). Thus, if BTX affects the muscarinic receptors through binding to the Na^+ channel, its effects should be antagonized by these two drugs. Indeed, the effect

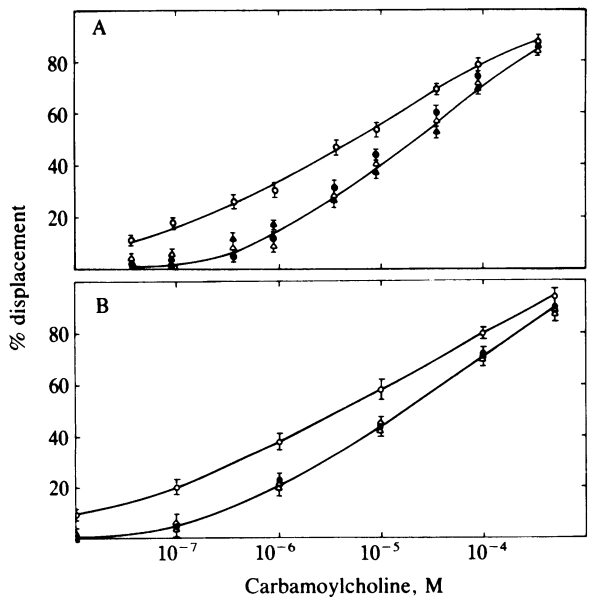

FIG. 3. Effects of veratridine (*A*) and local anesthetics (*B*) on the modulation of carbamoylcholine binding by BTX. Inhibition curves of the binding of 2 nM [^{3}H]4NMPB by carbamoylcholine to brainstem preparations are shown. (*A*) Displacement of [^{3}H]4NMPB by carbamoylcholine in the absence (●) and presence of 1 μM BTX (○), 200 μM veratridine (▲), and 1 μM BTX/200 μM veratridine (△). (*B*) Displacement of [^{3}H]4NMPB by carbamoylcholine alone (●) or in the presence of 1 μM BTX (○), 1 μM BTX/5 μM dibucaine (▲), and 1 μM BTX/5 μM tetracaine (△). Values shown are means ± SEM of three experiments; each experiment was performed in triplicate.

of BTX on carbamoylcholine binding to brainstem membrane preparations was completely blocked by 10 μM aconitine (not shown) or 200 μM veratridine (Fig. 3*A*). At these concentrations, the latter two drugs had no effect on either [^{3}H]-4NMPB or carbamoylcholine binding in the absence of BTX. In contrast to aconitine and veratridine, 0.3 μM tetrodotoxin [which acts at another site on the Na^+ channel (9–12)] had no effect on either agonist or antagonist binding to muscarinic receptors in either the brainstem or ventricle preparations.

Inhibition of BTX Effect by Local Anesthetics. Previous studies indicated that the binding of local anesthetics promotes an allosteric inhibition of BTX binding (see refs. 12 and 16–19 and references therein), increasing the "off-rate" of BTX and shifting the sodium channel to an inactive form. Local anesthetics also interact with the muscarinic receptors (3–5, 7), and here we report on the interaction of BTX with the latter. It was, therefore, of interest to examine whether local anesthetics alter the effect of BTX on the binding of muscarinic agonists. In view of the fact that local anesthetics perturb the binding of both antagonists and agonists, quantitative estimates of their effect on the interactions of BTX with the binding of muscarinic agonists could be obtained only with those local anesthetics that do not affect significantly either antagonist or agonist binding at the drug concentration used. From the variety of local anesthetics available (reviewed in ref. 16), only dibucaine and tetracaine fulfill this condition. Thus, the IC_{50} values (concentration that inhibits response by 50%) for direct inhibition of binding to the muscarinic site by dibucaine were 60 μM ([^{3}H]4NMPB binding) and 30 μM (carbamoylcholine binding). The respective values for tetracaine were 35 μM ([^{3}H]4NMPB) and 10 μM (carbamoylcholine). The effect of these drugs on the interaction of BTX with agonist binding to the muscarinic receptors occurred at much lower concentrations. This effect was determined in brainstem preparations by measurements of carbamoylcholine binding in the presence of 1 μM BTX. In these experiments, we followed the reduction observed in the BTX effect on the IC_{50} of agonist binding (measured as in Fig. 1) due to the presence of various concentrations of the local anesthetics. The results (Fig. 3*B*) indicated that these two drugs cancel the effect of BTX (the leftward shift of the agonist competition curve had disappeared). The IC_{50} values for dibucaine and tetracaine on the BTX effect were 1 μM and 3 μM, respectively. These values are in excellent agreement with a recent study (16) that measured the inhibition of the binding of [^{3}H]BTX-B to brain vesicular preparations by 44 local anesthetic drugs; in this study, the IC_{50} values obtained were 1.4 μM for dibucaine and 3.4 μM for tetracaine.

DISCUSSION

In the present study, we have demonstrated that BTX interacts with the muscarinic system. These interactions result in enhanced affinity for the binding of certain agonists (i.e., carbamoylcholine and acetylcholine) to the receptors. The observation that BTX has no effect on antagonist binding indicates that direct competition by BTX for the muscarinic binding sites is highly unlikely. The latter conclusion is in agreement with the recent work of Daly and co-workers (16), who observed that classical muscarinic antagonists such as scopolamine and atropine have virtually no effect on the binding of [^{3}H]BTX. Only cholinergic antagonists with potent local anesthetic activity, such as dicyclomine and biperidin, could interfere with [^{3}H]BTX binding, an inhibition attributed to their local anesthetic nature (16).

The effect of BTX on muscarinic agonist binding was observed at neurotoxin concentrations that are active in opening the voltage-sensitive Na^+ channels (10, 12, 16–19). The concentrations of the local anesthetics that blocked the BTX effect on the muscarinic system were in the range

known to inhibit the activation of the Na^+ channel by BTX. Moreover, the interactions of BTX with Na^+ channels are known to be antagonized by the "partial agonists" aconitine and veratridine. These two drugs also antagonized the perturbation induced by BTX on the muscarinic complex. It should be noted that previous studies on the mode of interactions of neurotoxins with the Na^+ channel were conducted with N18 neuroblastoma cells (e.g., see ref. 11), synaptic nerve ending particles (17), or resealed membrane vesicles (16). On the other hand, in the current study we used whole homogenates of the various tissues. Further studies on the effect of BTX on the muscarinic receptors in intact cells or in purified preparations might well result in more pronounced effects.

The effects of BTX on the muscarinic system as described above indicate that in specific tissues the muscarinic receptors may interact with another component, most likely a Na^+ channel. On the basis of the data presented here, this interpretation seems more likely than the alternative explanation—i.e., that BTX interacts directly with the muscarinic receptor at a site distinct from the muscarinic ligand binding site. The observation that the interactions of BTX with the muscarinic receptors are detected in brainstem and ventricle preparations, but not in the cortex, suggests either that they are region specific, or that they are undetectable in the cortex merely because the latter exhibits a much lower population of high affinity sites than the former two regions. The latter possibility gains some support from the experiments conducted with p[NH]ppG (Table 1), indicating association between the high-affinity state of the receptor and the BTX effect.

Another point that deserves attention is that the muscarinic agonists differ in their sensitivity to the BTX effect. Thus, while the binding of carbamoylcholine and acetylcholine in the brainstem and ventricle is modulated by the neurotoxin, oxotremorine binding in the same tissues is not affected. A possible explanation for this phenomenon is that oxotremorine binds to the muscarinic binding site in a different mode than carbamoylcholine and acetylcholine, a difference that is reflected in its lack of ability to sense the changes induced in the high-affinity state of the receptor by BTX. This interpretation is in accord with recent kinetic studies in our laboratory (unpublished observations), which indicate that the interaction of oxotremorine (as reflected in the binding parameters) with the high-affinity sites is very different from that of carbamoylcholine and acetylcholine. In this context, one has to cite the recent report of Brown and Brown (20), who observed major differences in the effects of carbamoylcholine and oxotremorine on both cAMP formation and phosphoinositide hydrolysis in embryonic chicken heart cells. This promoted the suggestion that the receptor state associated with the inhibition of adenylate cyclase is the state common to the two agonists, while only carbamoylcholine is associated with the phosphoinositide response.

We have previously proposed a model (1, 21) for the interaction of the muscarinic receptors with other membrane entities. Along the same lines, we hypothesize that in specific tissues or brain regions the muscarinic receptors are capable of interacting with Na^+ channels. Such "cross-talk" between these systems may represent a fundamental property in signal transduction in the membrane.

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