Biochemical Pharmacology, Vol. 36, No. 7, pp. 991–994, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

INFLUENCE OF VERATRIDINE ON [^{3}H]-L-QUINUCLIDINYL BENZILATE ([^{3}H]QNB) BINDING IN MOUSE HINDBRAIN

JAMES E. MACK* and JOHN C. MATTHEWS
Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677, U.S.A.

(*Recieved* 22 *February* 1986; *accepted* 1 *July* 1986)

Abstract—The neurotoxin veratridine is well known for its ability to open sodium channels in neuronal and muscle tissues in micromolar concentrations. It has also been shown that veratridine is an inhibitor of the potent muscarinic receptor antagonist L-quinuclidinyl benzilate (QNB) at these concentrations. These findings prompted us to examine the relationships between action potential sodium channels and muscarinic receptors in a glass-fiber filtration assay for [^{3}H]QNB binding to mouse hindbrain membranes using agents known to affect interconversion of the affinity states in some muscarinic receptor populations, i.e. guanosine triphosphate (GTP) and magnesium (Mg^{2+}). The actions of the sodium channel antagonist tetrodotoxin (TTX) were also examined. Veratridine inhibited [^{3}H]QNB binding with a K_i value of approximately 2.5 μM. This inhibition exhibited a competitive mechanism at higher concentrations (5–10 μM), while showing an apparent non-competitive action at low concentrations (1 μM). Magnesium caused a parallel shift to the right in the inhibition curve with a 32% increase in the veratridine K_i. GTP caused a non-parallel shift to the left with the greatest displacement occurring at lower veratridine concentrations (2–5 μM). The addition of magnesium to GTP did not alter the action of GTP significantly. TTX (5 μM) caused a parallel shift of the veratridine inhibition curve to the right. In addition, TTX alone inhibited the binding of [^{3}H]QNB. Therefore, it appears that there may be more than one binding site for veratridine which may be linked to the muscarinic system and that these may be action potential sodium channels.

Current awareness of muscarinic actions consists of a broad range of responses including adenylate cyclase inhibition, guanylate cyclase activation, activation of cyclic nucleotide phosphodiesterase, activation of phosphoinositide breakdown, modulation of protein phosphorylation, opening and closing of potassium channels, opening of calcium channels and the mobilization of intracellular calcium [1]. It is generally held that there is more than one subtype of muscarinic receptor based on anatomical location, affinity labeling and functional criteria [2]. In addition, there are thought to exist different binding affinity states within a particular subtype [3]. Receptor affinity states can be modified by guanine nucleotides (i.e. GTP) and divalent cations (i.e. Mg^{2+}). GTP is known to induce conversion of the high-affinity receptor state to the low-affinity state for agonist binding [4]. This suggests that muscarinic receptors may be capable of interacting with guanine nucleotide regulatory proteins [1]. Magnesium has been shown to increase the potency of guanine nucleotides in regulating agonist binding, as well as increasing agonist binding potency in the absence of guanine nucleotides [5–7].

Different muscarinic receptor antagonists such as [^{3}H]-pirenzepine and [^{3}H]-L-quinuclidinyl benzilate ([^{3}H]QNB) have been demonstrated to possess different binding affinities in various regions of the brain [3]. It has been speculated that these differences may represent functional coupling to different effectors in light of the observation that GTP influences agonist binding to a lesser extent in the cerebral cortex and has a greater effect in the brainstem and cerebellum.

Whether or not muscarinic receptor activity can influence action potential sodium channels is subject to question. Veratridine, aconitine and batrachotoxin (BTX) are neurotoxins that have been shown to be specific for the activation of action potential sodium channels by acting at a common site on the sodium channel [8, 9]. Early reports indicated that there was no real effect of veratridine or veratrine (a crude extract containing veratridine) on the binding of antagonists to muscarinic receptors [10, 11]. Recently reports have demonstrated that BTX modifies muscarinic receptors by enhancing the binding affinity of certain agonists (i.e. carbamylcholine and acetylcholine) to muscarinic receptors [12]. However, BTX alone failed to have any effect on antagonist binding. This lack of effect on antagonist binding has also been demonstrated previously [13]. Our laboratory has shown veratridine and aconitine to be inhibitors of the muscarinic antagonist QNB [14].

The objective of this work was to characterize further the possible actions of ligands which traditionally have been considered to be specific for the action potential sodium channel on muscarinic receptor specific ligand binding.

MATERIALS AND METHODS

Sucrose, veratridine, and GTP were purchased from the Sigma Chemical Co., St. Louis, MO. Tetrodotoxin was purchased from Calbiochem, La Jolla, CA. [^{3}H]QNB was purchased from New England Nuclear, Boston, MA. Mice were obtained from

* Author to whom correspondence should be sent.

Charles River Breeding Laboratories, Wilmington, MA. All other chemicals were of reagent quality or the finest available.

Four male Swiss mice (25–40 g) were used for each experiment. The mice were decapitated, and their brains were quickly excised and placed in ice-cold 0.32 M sucrose, 0.01 M Tris–HCl, 0.001 M NaN_3, pH 7.5 (SB). Excess blood and meningeal tissue were gently washed away with a stream of buffer from a pasteur pipette. The hindbrains were separated on the basis of gross anatomical markers. The pooled hindbrains were homogenized in 20 vol. of SB with a glass–Teflon homogenizer (five strokes). The homogenate was centrifuged at 1,000 *g* for 10 min at 0°. The supernatant fraction was recentrifuged at 28,000 *g* for 20 min at 0°. This step was repeated two more times. The pelleted membranes were resuspended to a final protein concentration of 1 mg/ml as determined by the method of Lowry *et al.* [15]. Aliquots of membranes (0.6 ml) were placed in siliconized test tubes (12 × 100 mm) and incubated with either tetrodotoxin or GTP for 15 min at room temperature. Veratridine solutions or ethanol controls (50 μl) were then added to each tube (100 pM [^{3}H]QNB for inhibition curves and 20–100 pM [^{3}H]QNB for Scatchard plots) and incubated for an additional 15 min at room temperature. [^{3}H]QNB (33.1 Ci/mmole) was then added to each tube and incubated for 60 min at room temperature. The final volume of each sample was 5 ml. Non-specific binding was determined by the addition of atropine sulfate at a final concentration of 10 μM. All experiments were run in triplicate. The incubations were terminated by filtering the membranes over glass-fiber filters (Fisher G-6, 12.1 cm) and washing with 5 ml of ice-cold SB. The filters were placed in scintillation vials, dark adapted, and equilibrated overnight with scintillation fluid (Scintiverse II, Fisher) and counted in a liquid scintillation counter. Experimental values were calculated using the BIOSOFT (Elsevier) software package. Apparent K_i values were generated using the Cheng–Prusoff equation [16]. Experimental values were compared to controls, and data were analyzed using a one-way ANOVA. $P \geq 0.05$ was considered significant.

RESULTS

Veratridine inhibited the binding of [^{3}H]QNB to mouse hindbrain membranes with a K_i value of approximately 2.5 μM. The inhibition of [^{3}H]QNB by veratridine exhibited both competitive and non-competitive components (Fig. 1). At low concentrations of veratridine (1–5 μM), the B_{max} for [^{3}H]QNB binding decreased 12% without any change in affinity. At higher concentrations of veratridine (5 and 10 μM) the K_d for [^{3}H]QNB binding increased by 68 and 126%, respectively, with no change in the B_{max}.

Magnesium (1 mM) caused a parallel shift to the right in the dose–response curve of veratridine inhibition of [^{3}H]QNB binding with a 32% increase in the K_i for veratridine (Fig. 2). In the absence of veratridine, magnesium decreased the binding of [^{3}H]QNB by 18%.

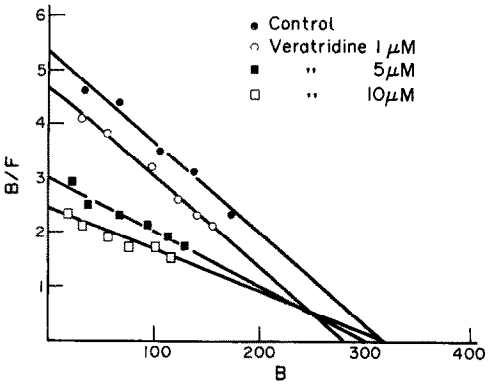

Fig. 1. Scatchard analysis of veratridine inhibition of [^{3}H]QNB binding. Control: B_{max} 314 fmoles/mg protein, and K_d 58.9 pM; veratridine 1 μM: B_{max} 278 fmoles/mg protein and K_d 59.2 pM; veratridine 5 μM: B_{max} 297 fmoles/mg protein, and K_d 98.9 pM; veratridine 10 μM: B_{max} 315 fmoles/mg protein, and K_d 133.4 pM. Data are from pooled hindbrains of four mice. Each point was run in triplicate.

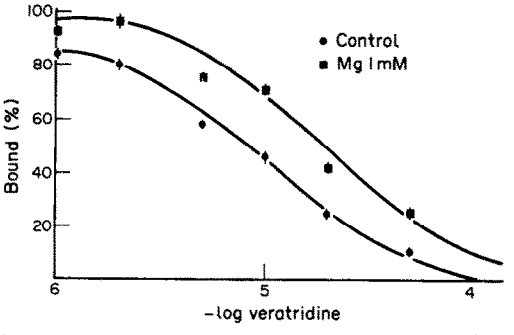

Fig. 2. Effect of magnesium on veratridine inhibition of [^{3}H]QNB (100 pM). Control: K_i = 2.5 μM; magnesium, K_i = 3.3 μM. Data are from pooled hindbrains of four mice and represent mean ±SEM, N = 3.

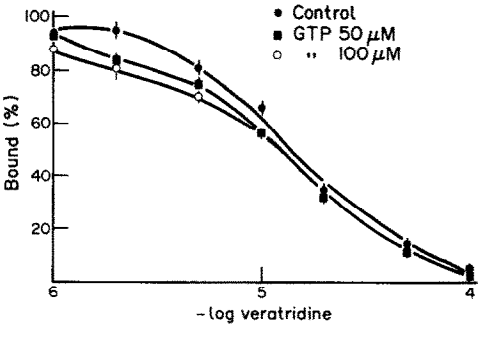

Fig. 3. Effect of GTP on veratridine inhibition of [^{3}H]QNB (100 pM). Control: K_i = 4.9 μM; GTP 50 μM: K_i = 3.8 μM; GTP 100 μM: K_i = 3.8 μM. Data are from pooled hindbrains of four mice and represent mean ±SEM, N = 3.

Guanosine triphosphate (50 or 100 μM) produced an enhancement of the veratridine inhibition of [^{3}H]QNB binding by approximately 15% but only at the lower concentrations of veratridine (2–5 μM) (Fig. 3). In the absence of veratridine, GTP (100 μM)

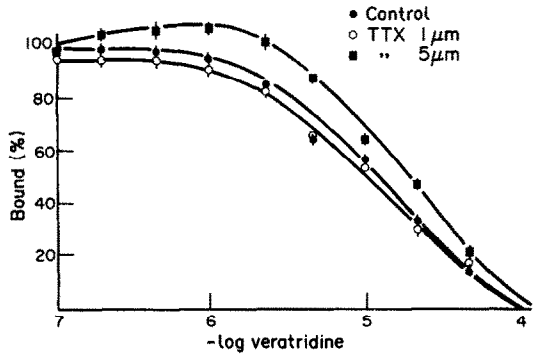

Fig. 4. Effect of TTX on veratridine inhibition of [^{3}H]QNB (100 pM). Control: K_i = 4.0 μM; TTX 1 μM: K_i = 3.8 μM; TTX 5 μM: K_i = 9.9 μM. Data are from pooled hindbrains of four mice and represent mean ±SEM, N = 3.

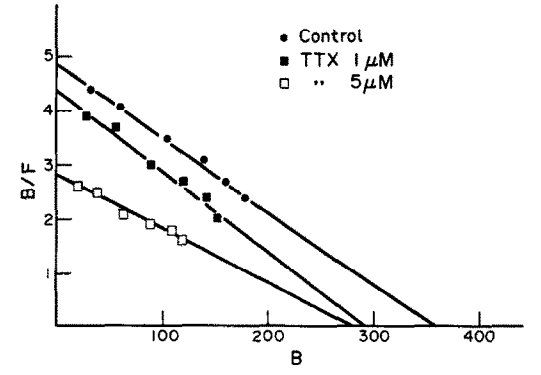

Fig. 5. Scatchard analysis of TTX inhibition of [^{3}H]QNB binding. Control: B_{max} 348 fmoles/mg protein, and K_d 69.9 pM; TTX 1 μM: B_{max} 288 fmoles/mg protein, and K_d 65.0 pM; TTX 5 μM: B_{max} 283 fmoles/mg protein, and K_d 102.0 pM. Data are from pooled hindbrains of four mice. Each point was run in triplicate.

increased the total binding of [^{3}H]QNB by approximately 10%. The addition of magnesium to GTP did not alter the effect of GTP.

Tetrodotoxin (5 μM) shifted the entire veratridine inhibition curve to the right with a 148% increase in the K_i (Fig. 4). Tetrodotoxin also inhibited [^{3}H]QNB binding in the absence of veratridine. This inhibition appeared to have both non-competitive and uncompetitive components (Fig. 5). At 1 μM, tetrodotoxin decreased the B_{max} of [^{3}H]QNB binding by 17% with no change in affinity, while at 5 μM, TTX decreased B_{max} by 19% and increased the K_d of [^{3}H]QNB binding by 46%.

DISCUSSION

The possible interactions betwen specific sodium channel ligands with muscarinic receptors is an area of growing interest. It has been shown that the sodium channel agonist batrachotoxin interacts with the muscarinic system causing an enhancement of cholinergic agonist binding with no effect on the binding of some cholinergic antagonists [12]. The cholinergic antagonists dicyclomine, biperiden and ditran have been shown to be relatively potent inhibitors of [^{3}H]BTX binding, whereas the classic antagonists atropine and scopolamine are without effect [13]. The neurotoxins veratridine and aconitine have been shown to compete with BTX binding in studies using cholinergic agonists, but by themselves have no effect [12]. In the same study, the sodium channel antagonist tetrodotoxin was also shown to be without effect. We have shown that veratridine, aconitine and tetrodotoxin can influence the binding of QNB in mouse forebrain [14].

In the present study, we demonstrated that veratridine and tetrodotoxin were capable of influencing the binding of QNB in mouse hindbrain. The hindbrain of the rat has been shown to possess a distinct population of receptors that are relatively insensitive to the binding of the specific muscarinic antagonist pirenzepine [3, 17, 18]. In addition, these binding sites are thought to interconvert from the high-affinity binding state to the low-affinity state in the presence of GTP [19, 20]. Therefore, the hindbrain should provide a good model to study the effect of agents on muscarinic receptor binding that can be influenced by guanine nucleotides. The effects of these agents on muscarinic receptor binding were in the same concentration ranges in which they actively influence action-potential sodium channels [20, 21].

Low concentrations of veratridine (1–5 μM) decreased the total binding of [^{3}H]QNB without influencing the apparent affinity of QNB, whereas higher concentrations of veratridine (10 μM) decreased the apparent affinity of QNB without effect on the B_{max}. It is not totally clear to us why higher concentrations of veratridine would reverse the effect of low veratridine concentrations on B_{max}. There may be multiple binding sites for veratridine that can influence one another. Agents that can induce interconversion of affinity sites in the muscarinic system (i.e. GTP and Mg^{2+}) influenced these abilities of veratridine to inhibit QNB binding. GTP enhanced the ability of low concentrations of veratridine to decrease total binding without influencing the inhibitory action of higher veratridine concentrations. This gives further support to the possibility of multiple binding sites for veratridine and may help to explain the changes in B_{max} at only low concentrations of veratridine. Mg^{2+} decreased the ability of veratridine to inhibit QNB binding at all concentrations.

The sodium channel antagonist tetrodotoxin was also demonstrated to influence the inhibition of [^{3}H]QNB binding by veratridine, causing a greater than 2-fold decrease in apparent [^{3}H]QNB affinity. In addition, tetrodotoxin alone inhibited [^{3}H]QNB binding with both competitive and non-competitive components. Because veratridine and tetrodotoxin are known to bind to different sites on the sodium channel and because they appear to inhibit [^{3}H]QNB binding by different mechanisms, it is unlikely that they are binding to the acetylcholine binding site. It is more likely that they may be acting at allosteric sites on the muscarinic receptor or as part of a ternary complex between the muscarinic receptor and the sodium channel.

The precise mechanisms of the influence of muscarinic activity on sodium channel function remain

unclear. It is apparent, however, that agents known to specifically modify ion flux through sodium channels can alter the binding of the muscarinic receptor antagonist quinuclidinyl benzilate and that the conformational state of the muscarinic receptor affects this alteration in binding. It is, therefore, reasonable to speculate that there may indeed be functional coupling between muscarinic acetylcholine receptors and action potential sodium channels.

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