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Positive Cooperativity in the Binding of Alcuronium and *N*-Methylscopolamine to Muscarinic Acetylcholine Receptors

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SUMMARY

The effect of the neuromuscular blocker alcuronium on the binding of *N*-[^{3}H]-methylscopolamine ([^{3}H]NMS) and l-[^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) to muscarinic binding sites in rat heart atria, longitudinal smooth muscle of the ileum, cerebral cortex, cerebellum, and submaxillary glands was measured using filtration techniques. In the presence of 10^{-5} M alcuronium, the binding of [^{3}H]NMS (which was present at a subsaturating concentration of 2×10^{-10} M) was increased 5.3-fold in the atria and smooth muscle and 3-fold in the cerebellum; no increase was observed in the brain cortex and salivary glands. The binding of [^{3}H]NMS was inhibited at 10^{-3} M and higher concentrations of alcuronium. The rates of [^{3}H]NMS association to and dissociation from muscarinic binding sites in the atria were diminished by 10^{-5} M alcuronium. Scatchard plots of [^{3}H]NMS binding data obtained with and without 10^{-5} M alcuronium indicated that the maximum number of binding sites was not altered by the drug, whereas the apparent K_d for [^{3}H]NMS was diminished. In contrast to [^{3}H]NMS, the effects of alcuronium on the binding of [^{3}H]QNB were only inhibitory. The concentration of alcuronium required to diminish the binding of [^{3}H]QNB by 50% (IC_{50}) was 4–7 μM in the atria, ileal smooth muscle, and the cerebellum, 140 μM in the brain cortex, and 1200 μM in the parotid gland. The results suggest that the binding of low concentrations of alcuronium to muscarinic receptors in the heart, ileal smooth muscle, and cerebellum allosterically increases the affinity of muscarinic receptors towards [^{3}H]NMS, although not [^{3}H]QNB. At high concentrations, alcuronium inhibits the binding of muscarinic ligands, presumably by competition for the classical muscarinic binding site. Positive cooperativity induced by alcuronium appears to be specific for the m2 (cardiac) subtype of muscarinic receptors.

Several neuromuscular blocking drugs (i.e., nicotinic antagonists) are known to interfere with the binding of specific ligands to muscarinic receptors. Among the drugs concerned, the interaction of gallamine with muscarinic receptors was investigated most systematically and was evaluated as competitive (1), allosteric (2–4), or both competitive and allosteric (5–9). In pharmacological experiments, the antimuscarinic effect of gallamine was found to be cardioselective (10) (see Refs. 11–13 for review), although cardioselectivity could not be revealed in radioligand binding experiments (14, 15) (but see Ref. 3); other evidence suggests that gallamine preferentially interacts with the pirenzepine-insensitive M_2 (as opposed to M_1) subtype of muscarinic receptors (8, 16).

Further investigation of the interaction of neuromuscular blocking drugs with muscarinic receptors may contribute to the understanding of differences between the subtypes of these receptors and of the regulation of their binding properties. In the present paper, we report observations showing that the binding of a classical muscarinic ligand, NMS, can be not only inhibited but also enhanced by alcuronium, one of the family of neuromuscular blocking agents (see Refs. 7 and 14 for information reported earlier on the interaction of alcuronium with muscarinic receptors).

The ability of alcuronium to induce positive cooperativity appears to be specific with regard to the ligand used and to the subtype of the receptor involved. Preliminary results of the present study have been reported at scientific meetings (17, 18).

Materials and Methods

Sources of drugs and reagents. Alcuronium chloride was obtained as a pure substance from Hoffmann-La Roche (Basel, Switzerland), [*methyl*-^{3}H]NMS from New England Nuclear (Dreieich, FRG), l-[^{3}H]QNB from the Radiochemical Centre (Amersham, England) and from New England Nuclear (Boston, MA), and HEPES from Sigma (St. Louis, MO).

Preparation of tissues. Female Wistar rats of 180–220 g of body mass were sacrificed by cervical dislocation and exsanguination. Heart atria, brain cortex, and cerebellum were homogenized in a Potter-Elvehjem all-glass homogenizer or a Polytron type homogenizer, in a 10-fold volume of an ice-cold homogenizing medium of the following composition (in mM): NaCl, 136; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1; Na_2HPO_4, 1; Na-HEPES (pH 7.4), 10; in experiments with longitudinal muscles

ABBREVIATIONS: NMS, N-methylscopolamine; QNB, quinuclidinylbenzilate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

of the ileum (19) and submaxillary glands, the homogenization medium was modified in that $CaCl_2$ and $MgSO_4$ were replaced by NaCl, and EDTA (0.5 mM) and phenylmethylsulfonyl fluoride (0.1 mM) were added. Experiments were performed on freshly prepared homogenates of the brain cortex, cerebellum, and submaxillary glands. In the work with the heart atria and ileal smooth muscle, tissues from several animals were pooled and the homogenates were first diluted with the homogenizing medium and then centrifuged for 10 min at 700 × *g*; the sediments were discarded and the supernatants (containing 50–75% of the [^{3}H]QNB binding sites present in the homogenates before centrifugation) were divided into small portions and kept at −20° for experiments performed within 4 weeks.

Incubations. Incubations were at 38°. In experiments with the atria, brain cortex, and cerebellum, the incubation medium was of the same composition as the homogenization medium. In experiments with ileal smooth muscles and salivary glands (homogenized in the absence of Ca^{2+} and Mg^{2+}), $CaCl_2$ and $MgSO_4$ were added to a final concentration of 2 and 1 mM, respectively; the final concentration of EDTA was 0.125 mM and that of phenylmethylsulfonyl fluoride was 0.025 mM. Alcuronium and/or atropine sulfate were added as indicated in Results.

In experiments with [^{3}H]NMS, the incubation volume was 0.8 ml (0.2 ml where indicated); the concentration of [^{3}H]NMS was in the range of 40–8000 pM in saturation experiments (for Scatchard plots) and 200 pM in experiments investigating the time course of [^{3}H]NMS binding and dissociation and the stimulation or inhibition of binding by alcuronium. Tissue homogenates were added in volumes ensuring a 50–100 pM concentration of [^{3}H]NMS binding sites. In experiments with [^{3}H]QNB, the incubation volume was 2 ml and the concentration of [^{3}H]QNB was 10–1000 pM in saturation experiments and 100, 30, or 20 pM in experiments on the inhibition of [^{3}H]QNB binding; the concentration of [^{3}H]QNB binding sites was close to 15 pM.

Incubations were started by addition of the homogenate to the incubation medium and lasted 180 min in competition (displacement) experiments; the Scatchard plots were based on incubations lasting 60 min. The incubation was stopped by addition of 5 ml of sodium phosphate buffer (10 mM, pH 7.4) into the incubation tubes with [^{3}H]NMS and subsequent filtration on Whatman glass fiber GF/C filters or by pouring of the content of the tubes with [^{3}H]QNB directly onto the filters. Filters were washed with 3 × 5 ml (experiments with [^{3}H]NMS) or 4 × 5 ml (experiments with [^{3}H]QNB) of sodium phosphate buffer, and the radioactivity retained on them was counted in Bray's scintillation solution in a Beckman LS 7800 scintillation counter. Centrifugation was used instead of filtration to separate free and bound ligand in one experiment. In this experiment, the incubation was in plastic microtubes and was finished by a 5-min centrifugation at 14,000 × *g*; the sediment was washed twice on its surface, dissolved in Hyamine, and used for scintillation counting in Bray's solution. Nonspecific binding of labeled ligands was determined in the presence of 1 μM atropine in all experiments.

[^{3}H]NMS receptor association and dissociation. The conditions used in measurements of [^{3}H]NMS association to and dissociation from muscarinic receptors were similar to the conditions used in experiments on the inhibition of [^{3}H]NMS binding; the concentration of [^{3}H]NMS was 200 pM. In experiments on association, homogenates and incubation media were prewarmed at 38°. In experiments on [^{3}H]NMS dissociation, the arrangement was similar to that described earlier for the dissociation of [^{3}H]QNB (7). The homogenate was incubated with 200 pM [^{3}H]NMS for 40 min at 38°, after which atropine sulfate (final concentration, 1 μM) was added, either alone or together with alcuronium (final concentration, 10 μM). The amounts of [^{3}H]NMS-receptor complexes measured by filtration at various times after the addition of atropine were expressed as a percentage of the amount of [^{3}H]NMS-receptor complexes present at the moment when atropine had been added.

Calculations of binding constants. Linear regression based on a least squares method was used to calculate K_d and B_{max} values from Scatchard plots and n_H and IC_{50} values from Hill plots (20). The procedure of Ehlert (21) was used to calculate the estimates of K_d values for the binding of alcuronium to the allosteric sites and of the cooperativity factor α.

Results

Effect of Increasing Concentrations of Alcuronium on the Binding of [^{3}H]QNB at Equilibrium

When the homogenates of the heart atria, longitudinal muscle of the ileum, submaxillary glands, cerebellum, and brain cortex were incubated in the presence of 100 pM [^{3}H]QNB, the amount of [^{3}H]QNB specifically bound to the membranes at the end of 3-hr incubations was progressively diminished if increasing concentrations of alcuronium had been added to the medium (Fig. 1). The concentrations of alcuronium needed to produce 50% inhibition of [^{3}H]QNB binding were very similar in the atria, smooth muscle, and cerebellum, whereas the concentration required in the brain cortex was 25 times and that in the salivary gland 359 times higher than in the atria (Table 1). Hill slopes were in the range of 0.49–0.68, clearly lower than unity in all tissues examined.

After the discovery that alcuronium increased the binding of [^{3}H]NMS (see below), we repeated the experiments concerning the effect of alcuronium on the binding of [^{3}H]QNB to the heart atria and cerebellum at lower concentrations of the radioligand (30 pM for the atria and 20 pM for the cerebellum, instead of 100 pM), ensuring lower occupancy under basal conditions. As shown in Fig. 2, the binding of [^{3}H]QNB was again not augmented by alcuronium at any concentration of the drug used. An additional experiment performed at 8 pM [^{3}H]QNB and with the incubation time extended to 300 min yielded similar results; alcuronium at 10^{-6} and 10^{-5} M inhibited the binding of [^{3}H]QNB by 36 and 72%, respectively.

Effect of Increasing Concentrations of Alcuronium on the Binding of [^{3}H]NMS at Equilibrium

When the homogenates were incubated in the presence of 200 pM [^{3}H]NMS and increasing concentrations of alcuronium, it was found that low concentrations of alcuronium increased the amount of [^{3}H]NMS bound to particles in the homogenates of the heart atria, ileal smooth muscle, and cerebellum, whereas no such increase was observed in brain cortex and salivary glands (Fig. 3). The greatest increase in [^{3}H]NMS binding was found at 10^{-5} M alcuronium; it was by 438, 444, and 203% in the heart atria, smooth muscle, and cerebellum, respectively. The binding of [^{3}H]NMS was diminished or blocked at high concentrations of alcuronium (>3×10^{-3} M). Results similar to those shown in Fig. 3 have been obtained in several additional experiments performed on the heart, ileum, and cerebellum by another experimenter, using 0.2-ml incubation volumes (data not shown).

Effect of Alcuronium on the Affinity and Number of Binding Sites for [^{3}H]NMS

To explain the mechanism by which 10^{-5} M alcuronium increased the binding of [^{3}H]NMS (which was present at a subsaturating concentration), one might assume that alcuronium increased either the number of binding sites available to [^{3}H]NMS or their affinity for the labeled ligand. Saturation experiments in which homogenates of the atria, ileal smooth muscle, and cerebellum were incubated with increasing concentrations of [^{3}H]NMS, with or without 10^{-5} M alcuronium,

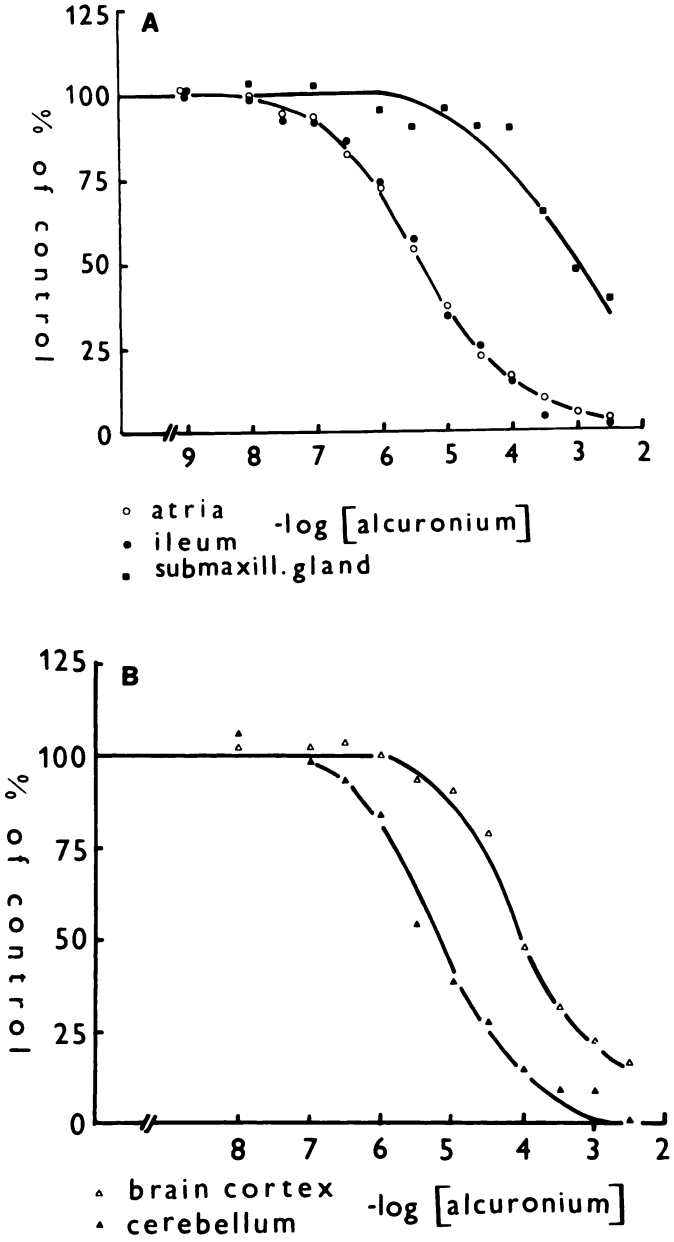


Fig. 1. Effect of alcuronium on the specific binding of [^{3}H]QNB to muscarinic binding sites. The concentration of [^{3}H]QNB was 100 pM and the incubations lasted 180 min. *Abscissa*, negative logarithm of the concentration of alcuronium (M). *Ordinate*, percentage of specific binding in the absence of alcuronium. *Top*, heart atria (○), longitudinal smooth muscle of the ileum (●), and submaxillary gland (■). *Bottom*, cerebellum (▲) and brain cortex (△). Individual *points* are means of six incubations for the ileum, five incubations for the submaxillary gland, and four incubations for the atria, brain cortex, and cerebellum. The *lines* were drawn by hand. IC_{50} and n_H values derived from these experiments are summarized in Table 1.

indicated that B_{max} was unchanged by alcuronium, whereas the affinity of the binding sites towards [^{3}H]NMS was augmented (Fig. 4 and Table 2). The stimulation of [^{3}H]NMS binding by alcuronium diminished progressively as the concentration of [^{3}H]NMS was increased. Alcuronium-induced decreases of K_d for the binding of [^{3}H]NMS were also observed in six additional experiments performed on the atria, ileum, and cerebellum by another experimenter, using 0.2-ml incubation volumes (data not shown).

TABLE 1

Effect of alcuronium on the binding of [^{3}H]QNB: IC_{50} values and Hill coefficients (n_H)

IC_{50} and n_H values were obtained from Hill plots of data obtained in experiments illustrated in Fig. 1, in which the homogenates were incubated with 100 pM [^{3}H]QNB and increasing concentrations of alcuronium; 95% confidence intervals are given in parentheses.

Tissue	IC_{50}	n_H
	μM	
Atrium	4.2 (3.4–5.4)	0.58 (0.51–0.65)
Ileal smooth muscle	5.0 (3.8–6.4)	0.62 (0.54–0.70)
Submaxillary gland	1510 (380–43,900)	0.49 (0.24–0.74)
Cerebellum	5.3 (1.3–13.0)	0.61 (0.36–0.86)
Cerebral cortex	106 (58–192)	0.68 (0.50–0.87)

Effect of Alcuronium on the Time Course of [^{3}H]NMS Association to and Dissociation from Receptors

In the absence of alcuronium, the binding of [^{3}H]NMS to atrial receptors proceeded rapidly, and apparent equilibrium was reached within less than 2 min (Fig. 5). In the presence of 10 μM alcuronium, the time needed to reach equilibrium was prolonged to about 45 min.

The effect of alcuronium on the dissociation of [^{3}H]NMS-receptor complexes is shown in Fig. 6. In the absence of alcuronium, the addition of atropine (1 μM) brought about rapid dissociation of more than 80% of [^{3}H]NMS-receptor complexes within 1 min; subsequent dissociation was slow. When alcuronium (10 μM) had been added simultaneously with atropine, only about 20% of [^{3}H]NMS-receptor complexes dissociated within the first minute and the rate of dissociation became extremely slow afterwards. On semilogarithmic plots (not shown), the dissociation of [^{3}H]NMS appeared to proceed in two exponential phases (a fast one and a slow one), both in the absence and in the presence of alcuronium; additional experiments are needed to clarify the kinetics of [^{3}H]NMS dissociation, taking into account data suggesting that slow isomerization of NMS-receptor complexes proceeds after NMS binding (22).

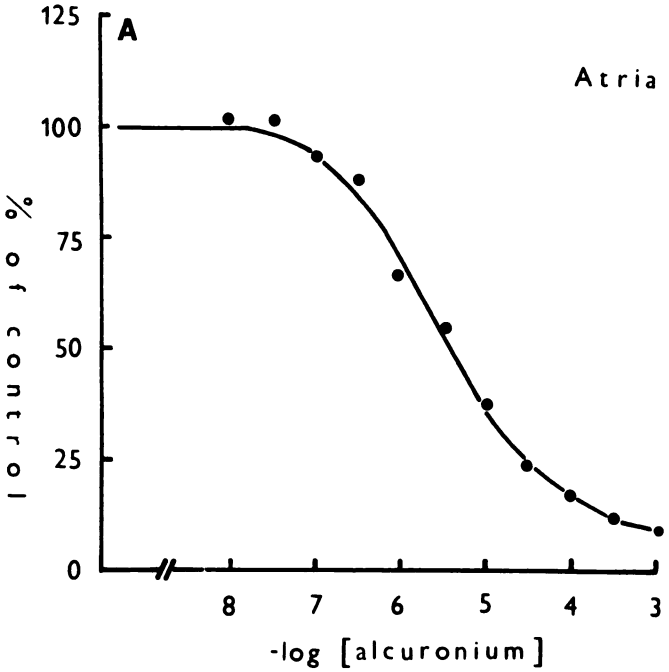
Estimates of K_d and Cooperativity Factors for Alcuronium

Ehlert (21) deduced ways in which apparent K_d values may be calculated for allosteric ligands in cases of positive or negative cooperativity, just as the magnitude of the cooperative interaction, expressed in terms of a cooperativity factor α. We have calculated these values by fitting our data in Figs. 1 and 2 (depression of [^{3}H]QNB binding by alcuronium) and Fig. 3 (stimulation of [^{3}H]NMS binding by alcuronium) to Eq. 6 in Ehlert's study. The estimates of K_d values (mean ± standard error) for the binding of alcuronium we obtained were 1.15 ± 0.20 μM (data for the atria in Fig. 1, experiments with the binding of 100 pM [^{3}H]QNB), 1.68 ± 0.24 μM (data for the atria in Fig. 2, experiments with the binding of 30 pM [^{3}H]QNB), and 1.13 ± 0.22 μM (data for the atria in Fig. 3, experiments with the binding of 200 pM [^{3}H]NMS). Corresponding cooperativity factor α values were 20.8 ± 5.6, 10.5 ± 1.7, and 0.135 ± 0.007, respectively.

Additional Experiments

Additional experiments were performed to make sure that the main results of the present work were not due to technical artifacts and to clarify minor points.

Dissociation of [^{3}H]NMS-receptor complexes at the end of incubation. Because alcuronium slows down the dissociation of [^{3}H]NMS from receptors, apparent increases in



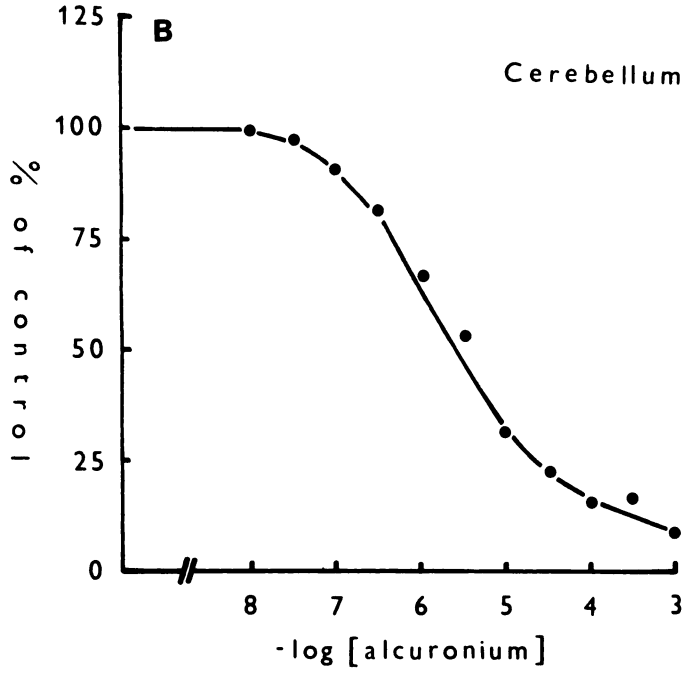
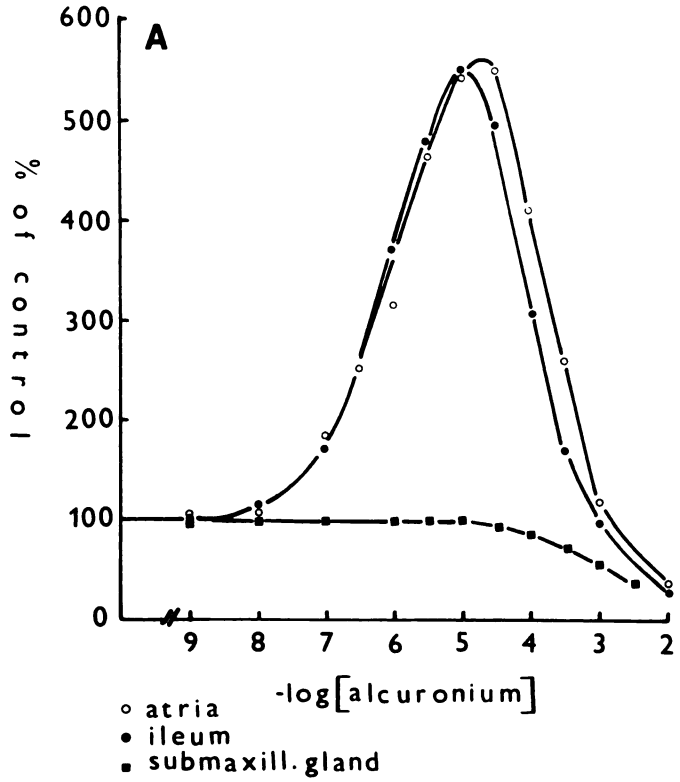


Fig. 2. Effect of alcuronium on the specific binding of [^{3}H]QNB to muscarinic binding sites in the atria (*top*) and cerebellum (*bottom*). The concentration of [^{3}H]QNB was 30 pM in experiments on atria and 20 pM in experiments on the cerebellum; other experimental conditions were the same as in Fig. 1. Individual *points* are means of 12–18 incubations for the atria and six incubations for the cerebellum. K_d values for the binding of [^{3}H]QNB (derived from Scatchard plots) were 60 pM in the atria and 30 pM in the cerebellum.

[^{3}H]NMS binding discovered in the presence of alcuronium might have been an artifact caused by the fact that fewer [^{3}H]NMS-receptor complexes dissociated during the time required for the separation procedure (addition of 5 ml of an ice-cold sodium phosphate buffer, filtration, and washing). We followed



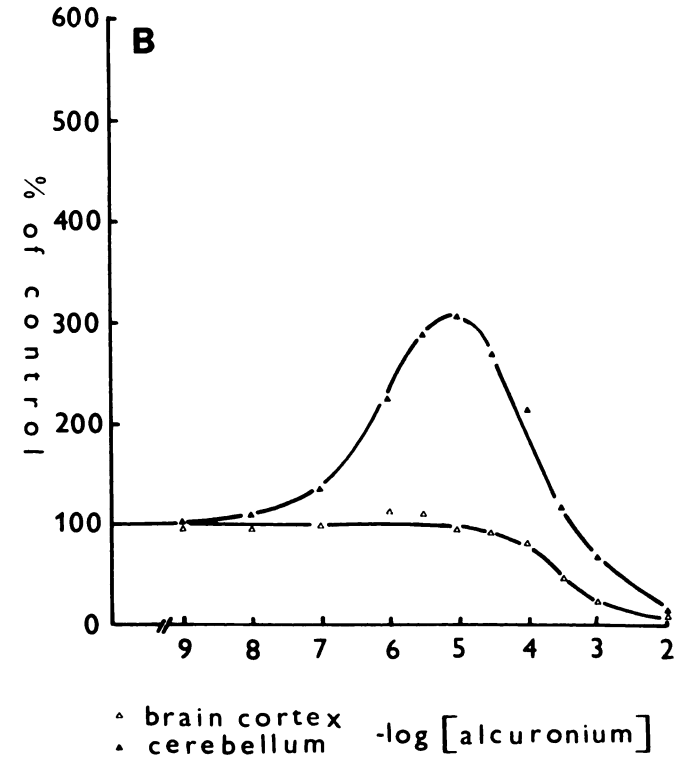


Fig. 3. Effect of alcuronium on the specific binding of [^{3}H]NMS to muscarinic binding sites. *Abscissa*, negative logarithm of the concentration of alcuronium (M). *Ordinate*, percentage of specific binding in the absence of alcuronium. *Top*, heart atria (○), longitudinal smooth muscle of the ileum (●), and submaxillary gland (■). *Bottom*, cerebellum (▲) and brain cortex (△). The concentration of [^{3}H]NMS was 200 pM and the

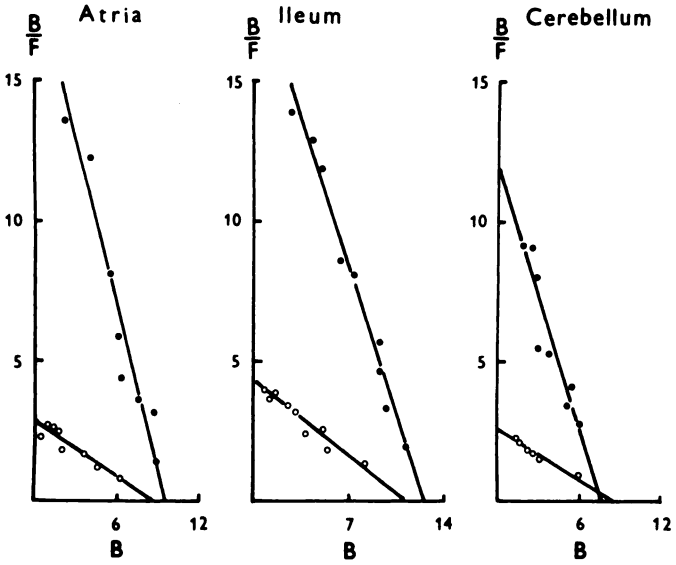


Fig. 4. Scatchard plots of [^{3}H]NMS binding data obtained in a representative experiment, in the absence (○) and presence (●) of 10 μM alcuronium. The concentrations of [^{3}H]NMS were in the range of 100–6500 pM and the incubations lasted 60 min. Individual *points* are the means of two incubations. The *lines* were obtained by linear regression. K_d and B_{max} values derived from this and another set of analogous plots are summarized in Table 2.

TABLE 2

Effect of alcuronium on the specific binding of [^{3}H]NMS: K_d and B_{max} values derived from Scatchard plots of data on the binding of [^{3}H]NMS in the absence and presence of 10 μM alcuronium

Data are means of two experiments, with incubations performed in duplicate; results of individual experiments are given in parentheses.

Tissue	K_D		$K_{d_{control}}/K_{d_{alcuronium}}$
	Control	Alcuronium	
	nM		
Atrium	3.58 (3.04; 4.12)	0.67 (0.52; 0.83)	5.3
Ileal smooth muscle	2.45 (2.63; 2.27)	0.75 (0.66; 0.85)	3.3
Cerebellum	2.70 (3.33; 2.08)	0.77 (0.65; 0.90)	3.5
Tissue	B_{max}		$B_{max_{control}}/B_{max_{alcuronium}}$
	Control	Alcuronium	
	pmol/g		
Atrium	8.7 (8.6; 8.8)	8.9 (9.6; 8.2)	0.98
Ileal smooth muscle	11.1 (11.2; 11.1)	13.1 (12.6; 13.6)	0.85
Cerebellum	8.1 (8.5; 7.8)	8.3 (7.6; 9.0)	0.98

the speed with which [^{3}H]NMS dissociates from receptors during this time interval, after the addition of 5 ml of cold phosphate buffer, and found that 14% of the bound ligand dissociated during 2 min (Fig. 6). This corresponded to a half-time of dissociation of about 8.5 min. Consequently, only about 1% of specific binding could have disappeared during the 10-sec period required for the separation procedure.

Use of centrifugation for the separation of free and bound [^{3}H]NMS. Filtration and centrifugation methods for the separation of free and specifically bound [^{3}H]NMS were compared in one experiment on atrial homogenates. In this experiment, alcuronium (10^{-5} M) increased the specific binding

incubations lasted 180 min. Individual *points* for the atria, ileum, brain cortex, and cerebellum are means of six incubations and those for the submaxillary gland, five incubations. The *lines* were drawn by hand.

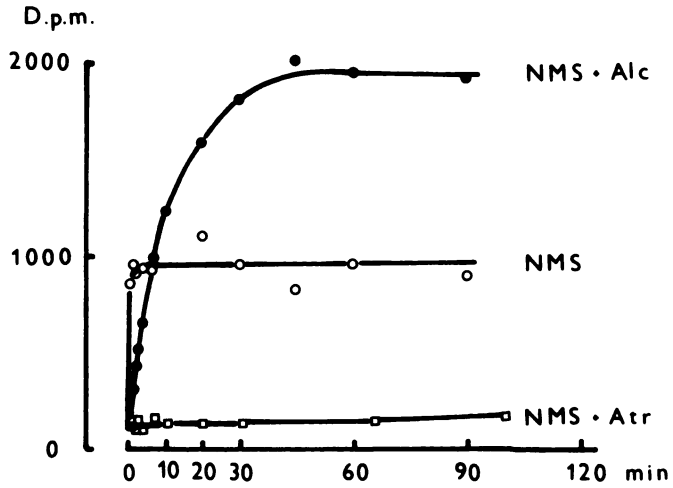


Fig. 5. Time course of [^{3}H]NMS binding to atrial homogenate (700 × *g* supernatant). *Lines* from the *top* to the *bottom*, [^{3}H]NMS (200 pM) plus alcuronium (10 μM) (●); [^{3}H]NMS alone (○); [^{3}H]NMS plus atropine (1 μM) (□). *Abscissa*, time (min). *Ordinate*, radioactivity retained on the filter (dpm). Data are from a representative experiment, with incubations performed in duplicate. The *lines* were drawn by hand.

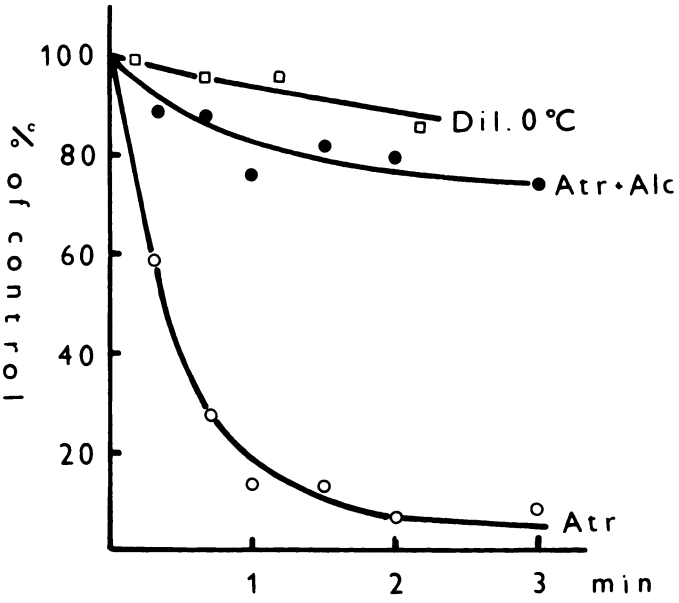


Fig. 6. Time course of the dissociation of [^{3}H]NMS-atrial receptor complexes (formed during a 40-min preincubation) after the addition of (from the *bottom* to the *top*) 1 μM atropine (○), 1 μM atropine plus 10 μM alcuronium (●), or 5 ml of ice-cold sodium phosphate buffer (□). *Abscissa*, time (min). *Ordinate*, percentage of undissociated [^{3}H]NMS-receptor complexes. Data are from a representative experiment, with incubations performed in duplicate. The *lines* were drawn by hand.

of [^{3}H]NMS measured at the end of a 180-min incubation 2.68 times with the filtration and 2.18 times with the centrifugation method.

Effect of alcuronium on [^{3}H]NMS binding in a mixture of cerebellar and cerebrocortical homogenates. As shown in Fig. 3, alcuronium (10^{-5} M) increased the binding of [^{3}H]NMS in cerebellar but not in cerebrocortical homogenates. If the difference between these tissues were due to the presence of an endogenous ligand or modifier in one of them, binding experiments on a mixture of homogenates might yield other than additive results. Table 3 indicates that this was not the case; the stimulatory effect of alcuronium (10^{-5} M) on [^{3}H]

TABLE 3

Effect of alcuronium on [^{3}H]NMS binding to a mixture of cerebellar and cerebrocortical homogenates during a 180-min incubation with 200 pM [^{3}H]NMS

Cerebellar homogenate in the mixture	Cerebrocortical homogenate in the mixture	[^{3}H]NMS binding in the presence of 10 μM alcuronium
%	%	% of control without alcuronium
0	100	110
25	75	128
50	50	147
75	25	166
100	0	204

NMS binding increased virtually linearly with the proportion of the cerebellar homogenate in the mixture.

Absence of an increase in free [^{3}H]NMS concentration produced by alcuronium. Conceivably, alcuronium might increase the specific binding of [^{3}H]NMS if it increased the concentration of free [^{3}H]NMS by displacing the radioligand from a nonspecifically bound pool. To check this possibility, we incubated atrial homogenates in the presence of 200 or 1000 pM [^{3}H]NMS and 1 μM atropine, with and without 10 μM alcuronium. After a 180-min incubation, the incubates were centrifuged for 45 min at 135,000 × *g*. The concentration of radioactivity discovered in the supernatant (free [^{3}H]NMS plus [^{3}H]NMS possibly bound to soluble proteins) was not measurably changed by alcuronium. Exclusion chromatography on Sephadex G-25 indicated that there was no measurable amount of [^{3}H]NMS bound to soluble proteins in the supernatants, either with or without alcuronium. Alcuronium was, thus, unlikely to act by increasing the concentration of free [^{3}H]NMS.

Discussion

Allosteric effects of neuromuscular blockers on the binding of specific ligands to muscarinic receptors have been known for some time (23, 24). Stockton *et al.* (3) explained the inhibition by gallamine of [^{3}H]NMS binding to cardiac muscarinic receptors as a result of negative cooperativity, and Dunlap and Brown (5) used a similar explanation for some of the inhibitory effects of gallamine on the binding of [^{3}H]QNB. At the same time, however, many effects of neuromuscular blockers on the binding of muscarinic ligands can be interpreted in terms of a simple competition (1, 8, 9). Allosteric interaction between neuromuscular blocking agents and classical muscarinic ligands is beyond doubt in experiments showing that certain neuromuscular blockers slow down the dissociation of preformed [^{3}H]QNB-receptor or [^{3}H]NMS-receptor complexes (3, 7, 9, 25, 26).

Although it is often difficult to distinguish the inhibition of binding due to competition from that due to strong negative cooperativity (27), the positive effect of alcuronium on the binding of [^{3}H]NMS described in the present work indicates unequivocally that the two substances bind to the receptor simultaneously and interact allosterically. The number of muscarinic binding sites in cardiac, ileal, and cerebellar homogenates remained unchanged in the presence of alcuronium, but the affinity of the receptors towards [^{3}H]NMS increased. Experiments demonstrating positive effects of alcuronium on the binding of [^{3}H]NMS had to be performed at low (unsaturating) concentrations of [^{3}H]NMS; it could be seen in saturation binding experiments that the stimulation of [^{3}H]NMS binding by alcuronium diminished as the concentrations of [^{3}H]NMS approached the saturating level. The conclusion that the number of sites available for [^{3}H]NMS binding was not increased by 10^{-5} M alcuronium is supported by the observation that no increase of [^{3}H]QNB binding could be discovered under the same conditions.

The finding that the increase in binding was only observed with [^{3}H]NMS but not with [^{3}H]QNB is not surprising, because allosteric interactions strongly depend on the chemical structure of the substances involved. Differences between the effects of gallamine on the dissociation of [^{3}H]NMS and of [^{3}H]QNB from the receptors have been noted (6, 9, 26).

Pharmacological and molecular genetic investigations permitted the identification of five types of muscarinic receptors, each of which represents an independent specific molecule (see Refs. 13, 28, and 29 for review). Using Bonner's (28) nomenclature of m1–m5 types, it seems most likely that the ability of alcuronium for positive cooperativity with the binding of [^{3}H]NMS is restricted to the m2 type; this type is present in the heart, smooth muscles, and some areas of the brain. Types m1 and m3, prevailing in the brain cortex and salivary glands (30–32), can safely be excluded as the targets of alcuronium, in view of the absence of the positive effects of the drug on the binding of [^{3}H]NMS to the brain cortex and the salivary gland. Type m4 seems unlikely as the target of alcuronium, because of its presence in the brain cortex (33, 34); type m5 is difficult to consider because of lack of clarity as to the site and extent of its expression (28, 35). In the smooth muscle of the ileum, most receptors are probably of the m2 type, but the m3 type is also present and functionally important (32, 36); in view of the absence of a positive effect of alcuronium on the binding of [^{3}H]NMS to the m3-rich salivary gland, we assume that the effect of alcuronium on the ileal smooth muscle only concerned the m2 population of smooth muscle receptors. We also assume that the effect of alcuronium on the binding in the cerebellum was due to the presence of the m2 type in the cerebellum, although the presence of m2 mRNA has not been, until now, specifically reported for this part of the brain; earlier reports have shown that muscarinic receptors prevailing in the cerebellum differ from those in the brain cortex by their low affinity for pirenzepine (37), a feature suggesting their relatedness to cardiac m2 receptors.

The nature of the binding site for alcuronium requires further study. In principle, two possibilities can be envisaged, namely, the binding site for alcuronium is entirely different from the muscarinic binding site or each receptor has two identical muscarinic binding sites and alcuronium binds to one of these sites. Data suggesting that muscarinic receptors are equipped with two muscarinic binding sites have been presented (38, 39) but received little further support; the view prevails that there is just one muscarinic binding site per receptor. Assuming that there is a specific allosteric binding site on muscarinic receptors, an intriguing question arises of what is its physiological ligand.

It seems worth noting that the concentration of alcuronium producing the maximum stimulation of the binding of [^{3}H]NMS to atrial, ileal, and cerebellar homogenates (10 μM) was very close to the concentrations of alcuronium producing 50% inhibition of the binding of [^{3}H]QNB in the same tissues (4.3–6.6 μM). K_d values for the binding of alcuronium calculated

according to the method of Ehlert (21) were even closer, 1.13 μM for binding to the site mediating the positive effect on the binding of [^{3}H]NMS and 1.15 or 1.68 μM for binding to the site mediating the negative effect on the binding of [^{3}H]QNB. This suggests that alcuronium exerted its positive effect on the binding of [^{3}H]NMS and its negative effect on the binding of [^{3}H]QNB via the same binding site, which should have been the allosteric site. This inference contrasts with the conclusion of Burke (8) that, in the case of gallamine, the inhibitory effect of its low concentrations is caused by its binding to the classical muscarinic site of brain muscarinic receptors. We assume that alcuronium only bound to the classical muscarinic sites at very high concentrations, which would explain the inhibition of [^{3}H]NMS binding observed at alcuronium concentrations of more than 10^{-4} M (cortex and salivary gland) or more than 10^{-3} M (atria, ileum, and cerebellum).

Although the concentrations of alcuronium producing the inhibition of [^{3}H]QNB binding were low in the heart, smooth muscle, and cerebellum, they were considerably higher in tissues with a low (or no) content of m2 muscarinic receptors, i.e., in the submaxillary gland and brain cortex. In the latter two tissues, the concentrations of alcuronium needed to inhibit the binding of [^{3}H]QNB by 50% were of the same order of magnitude as those inhibiting the binding of [^{3}H]NMS (10^{-3} M in the parotid gland and 10^{-4} M in the cortex). It is tempting to speculate that, in these tissues, the binding of both radioligands was prevented by the binding of alcuronium to the classical muscarinic site.

The discovery of positive cooperativity between the binding of [^{3}H]NMS and alcuronium is likely to facilitate further investigations of the control of the binding properties of muscarinic receptors and may open new approaches to the pharmacology of muscarinic synapses.

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