0026-895X/94/040709-09$03.00/0

MOLECULAR PHARMACOLOGY, 45:709–717

Mechanisms of Steric and Cooperative Actions of Alcuronium on Cardiac Muscarinic Acetylcholine Receptors

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Received August 4, 1993; Accepted December 6, 1993

SUMMARY

Kinetics of the interactions between the neuromuscular blocker alcuronium, the specific muscarinic antagonist *N*-[*methyl*-^{3}H] methyl scopolamine ([^{3}H]NMS), and muscarinic receptors were investigated in homogenates of rat heart atria. Two effects of alcuronium on the binding of [^{3}H]NMS could be distinguished. (a) Alcuronium concentration-dependently slowed the association of [^{3}H]NMS with receptors and the dissociation of [^{3}H]NMS from receptors so that, at high alcuronium concentrations, equilibrium binding could not be reached, even after 20 hr, without special precautions. (b) Alcuronium increased the affinity of receptors for [^{3}H]NMS, which was manifested by a decrease of the apparent K_d (>3-fold) with no change in the B_{max} for [^{3}H]NMS binding. The effects of alcuronium on the rates of [^{3}H]NMS association and dissociation can be explained only by a reaction mechanism in which [^{3}H]NMS binds only to free receptors (not occupied by alcuronium), whereas alcuronium binds both to free receptors and to receptors occupied by [^{3}H]NMS. Similarly, [^{3}H]NMS cannot dissociate from receptors as long as alcuronium is attached to them. Experimental data agree with corresponding mathematical models. It is proposed that alcuronium blocks entry to the pocket containing the [^{3}H]NMS binding site. In addition to this blocking effect, alcuronium has a positive allosteric effect on [^{3}H]NMS binding, presumably by inducing a conformational change of the orthosteric muscarinic binding site. Earlier observations suggesting that, at high concentrations, alcuronium also competes for [^{3}H]NMS binding sites can be explained by insufficient equilibration of the system.

A number of substances are known to affect the binding properties of muscarinic acetylcholine receptors by binding to an allosteric site that is different from the classical (orthosteric) binding site for acetylcholine, the physiological agonist of the receptors (see Refs. 1 and 2 for reviews). In spite of their obvious pharmacological importance, very little is known about the kinetics and mechanisms of allosteric interactions with muscarinic receptors. The main problem that hampered their clarification involved the fact that, until recently, all known allosteric agents inhibited the binding of muscarinic ligands to the orthosteric sites and suppressed muscarinic transmission, but it is very difficult to distinguish between inhibition that is competitive and that which is based on a strong allosteric action (3, 4).

Of all allosteric agents, gallamine and other neuromuscular blockers have been most thoroughly investigated for their negative actions on muscarinic receptors (5–17). We recently found that alcuronium, which belongs to the group of neuromuscular blockers, is able to increase the binding of [^{3}H]NMS to muscarinic receptors in the heart, cerebellum, and ileum, apparently exerting a positive allosteric action on orthosteric binding (18). The positive effect of alcuronium on [^{3}H]NMS binding indicates that both agents associate with the receptor simultaneously, with the formation of a ternary complex in which either ligand binds to a different site.

In the present study, we wish to present kinetic data providing information on the sequence of [^{3}H]NMS and alcuronium binding, to formulate a hypothesis explaining their interaction with the receptor, and to show correspondence between experimental findings and theoretical predictions. The assumptions that were necessary for building theoretical models are explained in the Appendix. Moreover, we wish to withdraw our earlier suggestion (18) that, at high concentrations, alcuronium also competes with [^{3}H]NMS for the orthosteric binding site on cardiac muscarinic receptors, in addition to allosteric binding. Conference abstracts of the present data have been published (19–22).

Materials and Methods

Sources of drugs and reagents. [*methyl*-^{3}H]NMS was obtained from DuPont-NEN (Dreieich, Germany), alcuronium was from Hoffmann-La Roche (Basel, Switzerland), and HEPES was from Sigma (St. Louis, MO).

Tissue preparation. Experiments were performed on homogenates of cardiac atria taken from male Wistar rats (220–250-g body mass)

This study was supported by Grant 309/93/0588 from the Grant Agency of the Czech Republic.

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

that were sacrificed by cervical dislocation and exsanguination. Atria from 10–20 rats were pooled and homogenized in a Potter-Elvehjem all-glass homogenizer, in a 10-fold volume of ice-cold aqueous homogenization medium containing (in mM) 136 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 Na_2HPO_4, and 10 Na-HEPES, pH 7.4. The homogenates were centrifuged for 10 min at 700 × *g*, the sediments were discarded, and the supernatants were diluted with the homogenization medium, divided into small portions, and kept frozen at −20° until the day of the experiment.

Incubations. Incubations were performed at 25°, in a total volume of 0.8 ml (in experiments with [^{3}H]NMS saturation) or 1.6 ml (in all other experiments). The basic composition of the incubation medium corresponded to that of the homogenization medium; atrial homogenates were added in volumes ensuring 50–100 pM concentration of the [^{3}H]NMS binding sites, whereas the concentration of added [^{3}H]NMS was in the range of 50–3200 pM in saturation binding experiments and 200 pM in all other experiments. The timing and sequence of the additions of [^{3}H]NMS and alcuronium were varied as described in Results. Nonspecific radioligand binding was determined in the presence of 5 μM atropine. Incubations were terminated by the addition of 5 ml of 10 mM sodium phosphate buffer, pH 7.4, and subsequent filtration on Whatman GF/C glass fiber filters, with two washes of the tubes and filters with 5-ml portions of phosphate buffer. The filters were dried and the radioactivity retained on them was counted in Bray's scintillation solution, in a scintillation counter.

Protein determination. Protein determination was according to the method of Peterson (23).

Calculations. Values of the apparent equilibrium dissociation constant for the binding of [^{3}H]NMS (K_d*), the maximum number of [^{3}H]NMS binding sites (B_{max}), the observed association rate constant (k^*_{on}), and the observed dissociation rate constant (k^*_{off}) were calculated by nonlinear regression (24).

Modeling. The derivation of equations necessary for theoretical modeling is described in the Appendix.

Results

Effect of alcuronium on the binding of [^{3}H]NMS to receptors. In the absence of alcuronium, the association of [^{3}H]NMS with muscarinic receptors (atropine-sensitive binding) proceeded rapidly and reached equilibrium within <8 min (as shown in Fig. 2). To investigate the effect of alcuronium, homogenates were preincubated with increasing concentrations of the drug and 200 pM [^{3}H]NMS was added after 2 hr of preincubation. As shown in Fig. 1, alcuronium had two effects on [^{3}H]NMS association. (a) The rate of association was slowed and equilibrium was reached only after approximately 1, 6, and 20 hr in the presence of 3×10^{-6}, 1×10^{-5}, and 3×10^{-5} M alcuronium, respectively. (b) The extent of binding (dpm/incubation tube) achieved at equilibrium was more than doubled in the presence of 3×10^{-6}, 1×10^{-5}, and 3×10^{-5} M alcuronium, compared with binding without the drug. It should be noted that, at long time intervals, binding was enhanced (compared with controls without alcuronium) even at the highest concentrations of drug used (1×10^{-4} and 3×10^{-4} M).

The time course of [^{3}H]NMS association in the presence of alcuronium corresponded to that expected for binding to a single population of binding sites. The values of observed association rate constants calculated from the experiments shown in Fig. 1 have been summarized in Table 1.

Fig. 2 shows that, even if [^{3}H]NMS was permitted to bind in the absence of alcuronium, subsequent addition of the drug brought about the binding of additional radioligand and the final binding was the same, independently of whether [^{3}H]NMS was added before or after alcuronium. Data for additional

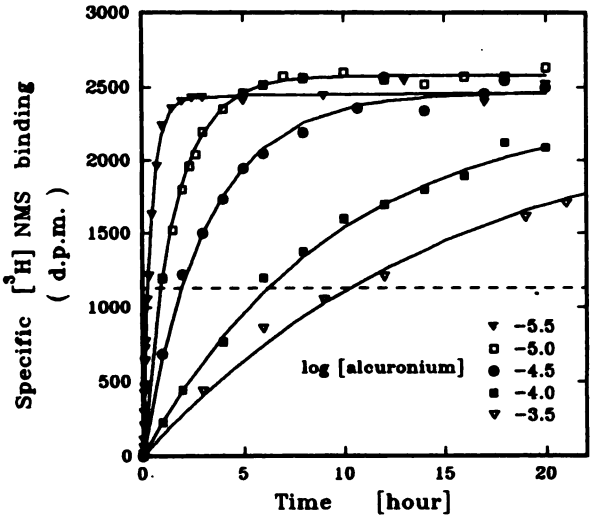

Fig. 1. Time course of the binding of [^{3}H]NMS to muscarinic binding sites in atrial homogenates in the presence of 3×10^{-6} to 3×10^{-4} M alcuronium. The homogenates were preincubated with the indicated concentrations of alcuronium for 2 hr. [^{3}H]NMS was then added at time 0, to a final concentration of 200 pM. The time course of control [^{3}H]NMS binding (without alcuronium) could not be shown on the time scale used because binding reached saturation within <10 min (but see Fig. 2). *Dashed horizontal line*, level of control binding at saturation, which was smaller than that achieved in the presence of alcuronium. Data are means of three experiments, each performed with triplicate incubations.

TABLE 1

Values of observed association rate constants (k^*_{on}) for the binding of [^{3}H]NMS to atrial membranes in the absence or in the presence of 3×10^{-6} to 3×10^{-4} M alcuronium

Data (mean ± standard error) have been calculated from experiments shown in Fig. 1.

Alcuronium concentration	k^*_{on}
M	$min^{-1} \times M^{-1}$
0	$7.27 \pm 0.09 \times 10^{-1}$
3×10^{-6}	$3.96 \pm 0.31 \times 10^{-2}$
1×10^{-5}	$1.04 \pm 0.15 \times 10^{-2}$
3×10^{-5}	$7.12 \pm 0.86 \times 10^{-3}$
1×10^{-4}	$4.03 \pm 0.04 \times 10^{-3}$
3×10^{-4}	$1.46 \pm 0.12 \times 10^{-3}$

[^{3}H]NMS binding induced by the addition of alcuronium (Fig. 2) were fitted best to the equation for binding to two sites, with 77% of radioligand binding being slow ($k^*_{on} = 4.90 \pm 0.09 \times 10^{-2}$ min^{-1} M^{-1}) and 23% of binding being fast ($k^*_{on} = 5.32 \pm 0.88 \times 10^{-1}$ min^{-1} M^{-1}).

Effect of alcuronium on the dissociation of [^{3}H]NMS from receptors. In experiments designed to observe the rates of [^{3}H]NMS dissociation from receptors, homogenates were first equilibrated with the radioligand and alcuronium, after which 5 μM atropine was added to prevent the reassociation of [^{3}H]NMS spontaneously dissociating from receptors. In the presence of high concentrations of alcuronium, the binding of [^{3}H]NMS proceeds very slowly, as shown in Fig. 1. To speed it up, a two-step preincubation was used; in the first step receptors were preincubated for 3 hr with 200 pM [^{3}H]NMS and 3×10^{-6} M alcuronium and in the second step the concentration of alcuronium was increased to the desired final level and the preincubation was continued for another 2 hr, after which atropine was added.

As shown in Fig. 3, alcuronium concentration-dependently

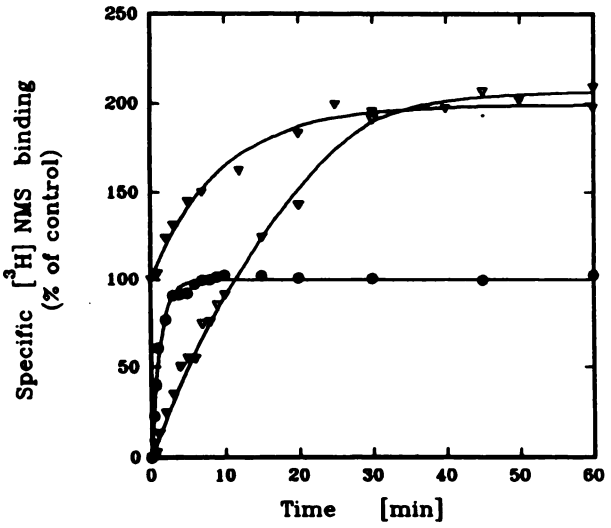

Fig. 2. Time course of the binding of [^{3}H]NMS to muscarinic binding sites in atrial homogenates, depending on the sequence of addition of alcuronium and the radioligand. Three paradigms were utilized. ●, [^{3}H]NMS (200 pM) was added at time 0 (*abscissa*) and no alcuronium was applied (control samples); ▽, homogenates were preincubated with 3×10^{-6} M alcuronium for 2 hr and 200 pM [^{3}H]NMS was added at time 0; ▼, homogenates were preincubated with 200 pM [^{3}H]NMS for 2 hr and 3×10^{-6} M alcuronium was added at time 0. Data are means of two experiments, each performed with triplicate incubations. Data on the *ordinate* are expressed as percentage of [^{3}H]NMS binding in samples without alcuronium that had reached equilibrium.

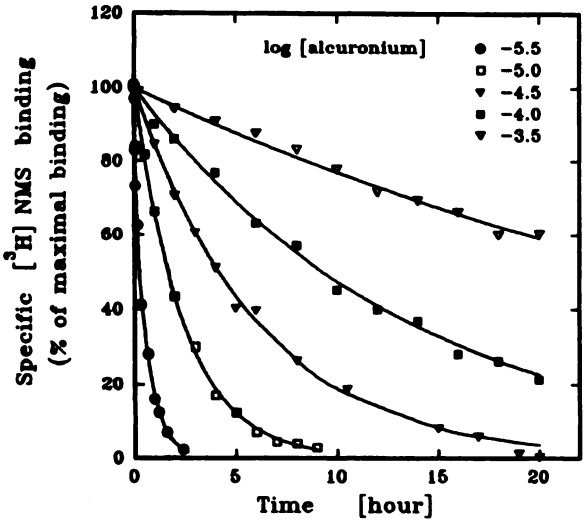

Fig. 3. Effect of increasing concentrations of alcuronium on the time course of atropine-induced dissociation of [^{3}H]NMS from receptors. To approximate equilibrium between receptors, [^{3}H]NMS, and alcuronium, samples were preincubated for a total of 5 hr with 200 pM [^{3}H]NMS. During the first 3 hr of preincubation, alcuronium was present at a concentration of 3×10^{-6} M in all samples. During the next 2 hr of preincubation, the concentration of alcuronium was augmented to reach the indicated values. At the end of the 5-hr preincubation, 5 μM atropine sulfate was added at time 0 (*abscissa*). The binding observed at individual time points after the addition of atropine is expressed as percentage of binding at time 0. Data are means of four experiments, each performed with triplicate incubations.

slowed [^{3}H]NMS dissociation, with the half-times for dissociation rising from 1.4 min in its absence to 2318 min with a concentration of 3×10^{-4} M. The best fit of the data was obtained with the assumption that dissociation occurred from a single population of binding sites at all except the lowest concentration (3×10^{-6} M) of alcuronium (Table 2). At 3×10^{-6} M alcuronium, 72% of bound [^{3}H]NMS appeared to dissociate at a slow rate and 28% at a fast rate (Table 2).

Effect of alcuronium on the affinity and number of [^{3}H]NMS binding sites. To overcome the problems arising from the slow binding of [^{3}H]NMS to receptors in the presence of high concentrations of alcuronium (Fig. 1), the following procedure was adopted for saturation binding (Scatchard-type) experiments. For 10 hr, homogenates were incubated in the presence of increasing concentrations of [^{3}H]NMS, either in the absence or in the presence of 3×10^{-6} M alcuronium. After 10 hr, the concentration of alcuronium was increased in those tubes in which it had to reach either 3×10^{-5} M or 3×10^{-4} M, and the incubation of all tubes was continued for another 10 hr. It was ascertained in preliminary experiments with control samples and samples with 3×10^{-6} M alcuronium that the same K_d* and B_{max} values were obtained after 3 hr or 20 hr of incubation (data not shown). As shown in Fig. 4 and Table 3, alcuronium concentration-dependently diminished the apparent equilibrium dissociation constant for the binding of [^{3}H]NMS, without altering the total number of binding sites.

In experiments described in Fig. 5, the effect of increasing concentrations of alcuronium on the binding of a fixed and nonsaturating (200 pM) concentration of [^{3}H]NMS was measured after incubations lasting 0.25, 3, or 20 hr. It was evident that the system was very slow in approaching equilibrium, which was apparently not attained even after 20 hr. High concentrations of alcuronium that appeared inhibitory with regard to [^{3}H]NMS binding at short incubation times became stimulatory at long incubation times; e.g., at 1×10^{-4} M alcuronium, the binding of [^{3}H]NMS corresponded to 5.4%, 93%, or 189% of control binding after incubations lasting 0.25, 3, or 20 hr, respectively. In Fig. 5, the binding of [^{3}H]NMS observed after 20 hr in the presence of 3×10^{-4} M alcuronium appeared considerably lower than that at 3×10^{-5} M alcuronium. The difference between these two concentrations of alcuronium did disappear, however, when the attainment of equilibrium was facilitated by appropriately arranged preincubation; in experiments in Fig. 4 (Scatchard-type experiments, involving 10-hr incubation with 3×10^{-6} M alcuronium followed by 10 hr with more elevated concentrations of alcuronium), the binding of [^{3}H]NMS achieved at 202–208 pM radioligand concentrations corresponded to 190% and 187% of control at 3×10^{-5} and 3×10^{-4} alcuronium, respectively.

Data points in Fig. 5 have been connected by dashed curves

TABLE 2

Values of apparent dissociation rate constants (k^*_{off}) for atropine-induced dissociation of [^{3}H]NMS from receptors, in the absence or in the presence of 3×10^{-6} to 3×10^{-4} M alcuronium

Data (mean ± standard error) have been calculated from experiments shown in Fig. 3. Except for experiments with 3×10^{-6} M alcuronium, the best fit was for simple exponential functions with single k^*_{off} values. For experiments with 3×10^{-6} M alcuronium, the best fit suggested that 23% of [^{3}H]NMS dissociated at a fast rate and 77% dissociated at a slow rate.

Alcuronium concentration	$k^*_{off_1}$	$k^*_{off_2}$
M	min^{-1}	min^{-1}
0	$5.00 \pm 0.08 \times 10^{-1}$	
3×10^{-6}	$2.03 \pm 0.13 \times 10^{-2}$	$3.9 \pm 0.56 \times 10^{-1}$
1×10^{-5}	$7.02 \pm 0.03 \times 10^{-3}$	
3×10^{-5}	$2.78 \pm 0.11 \times 10^{-3}$	
1×10^{-4}	$1.56 \pm 0.19 \times 10^{-3}$	
3×10^{-4}	$2.99 \pm 0.73 \times 10^{-4}$	

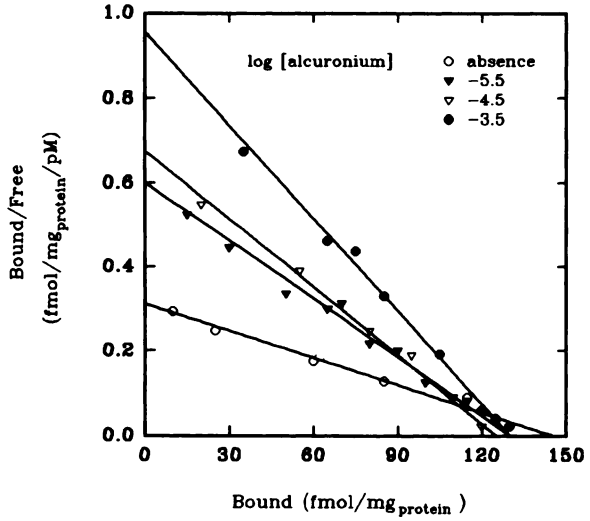

Fig. 4. Scatchard plots of [^{3}H]NMS saturation binding experiments performed in the absence or in the presence of 3×10^{-6} to 3×10^{-4} M alcuronium. To approximate equilibrium binding, atrial homogenates were preincubated for 10 hr with various concentrations of [^{3}H]NMS (50 to 3200 pM), either without alcuronium (control) or with 3×10^{-6} M alcuronium. After 10 hr, additional alcuronium was added to those samples in which its concentration had to reach either 3×10^{-5} or 3×10^{-4} M, and all samples were incubated for another 10 hr. Data are means of two experiments, each performed with triplicate incubations. Calculated values of B_{max} and apparent K_d have been summarized in Table 3.

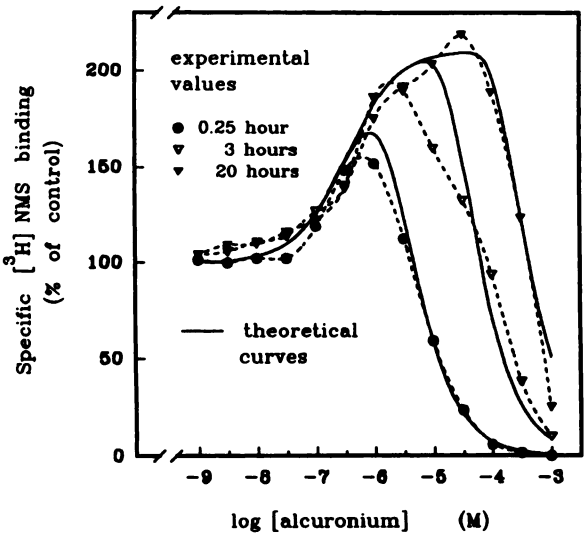

Fig. 5. Effect of increasing concentrations of alcuronium on the binding of 200 pM [^{3}H]NMS to atrial membranes during incubations lasting 0.25, 3, or 20 hr. Membranes were preincubated with the indicated concentrations of alcuronium for 2 hr and the radioligand was added at time 0. The specific binding with alcuronium is expressed as percentage of specific binding in corresponding samples without alcuronium, which was 958 dpm/filter after 0.25 hr, 1102 dpm/filter after 3 hr, and 974 dpm/filter after 20 hr. Data are means ± standard errors of six determinations (two experiments with triplicate incubations). The *dashed lines* connecting the experimental points have been drawn by polynomial interpolation. Superimposed on them are *continuous lines* that have been computed as described in Appendix C, for a model corresponding to the scheme in Fig. 6b, based on parameters listed in the legend to Fig. 8.

TABLE 3

Apparent K^*_d and B_{max} values for the binding of [^{3}H]NMS to atrial receptors in the absence and in the presence of alcuronium

Data (mean ± standard error) have been calculated by nonlinear regression from saturation binding experiments described in Fig. 4.

Alcuronium concentration	K^*_d	B_{max}
M	pM	fmol/mg of protein
0	467 ± 59	137 ± 6
1×10^{-6}	220 ± 21	129 ± 4
3×10^{-6}	197 ± 29	130 ± 6
3×10^{-5}	185 ± 31	125 ± 3
3×10^{-4}	136 ± 27	130 ± 3

drawn by polynomial interpolation. Superimposed on them are continuous curves predicted by the model given in the scheme in Fig. 6b and computed as described in Appendix C, based on the same parameters as those listed in the legend to Fig. 8.

To obtain reliable estimates of the dissociation constant of the receptor-alcuronium complex (K_{AR}) and of the cooperativity factor α, additional experiments were performed measuring the effect of 1×10^{-9} to 3×10^{-6} M alcuronium on the binding of [^{3}H]NMS (Fig. 7); within this range of alcuronium concentrations, equilibrium binding was safely attained during the incubation period used. Fitting data to eq. 6 of Ehlert (4) yielded the curve superimposed on the data in Fig. 7, which corresponds to K_{AR} = 667 nM and α = 0.237. Because by definition (4) $\alpha = K_{ARL}/K_{AR}$, these values suggest that the value of K_{ARL} (i.e., the equilibrium dissociation constant of the reversible reaction RL + A = ARL) is close to 158 nM.

Discussion

The main experimental findings of the present work may be summarized in just two points. (a) Alcuronium concentration-dependently slows both the rate of [^{3}H]NMS association with

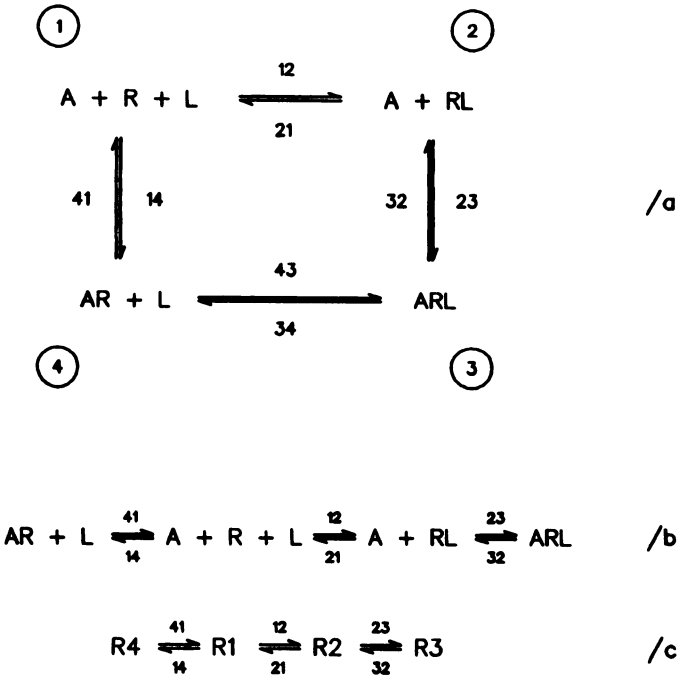

Fig. 6. Interactions between receptor R, classical ligand L, and allosteric ligand A. a, Fully independent and reversible binding of both ligands. b, Interactions under the condition that reactions 43 and 34, envisioned in a, cannot occur, i.e., L cannot bind to AR and cannot dissociate from ARL. c, Same as in b, but the four different states of R (R, RL, ARL, and RA) have been denoted by symbols R1–R4.

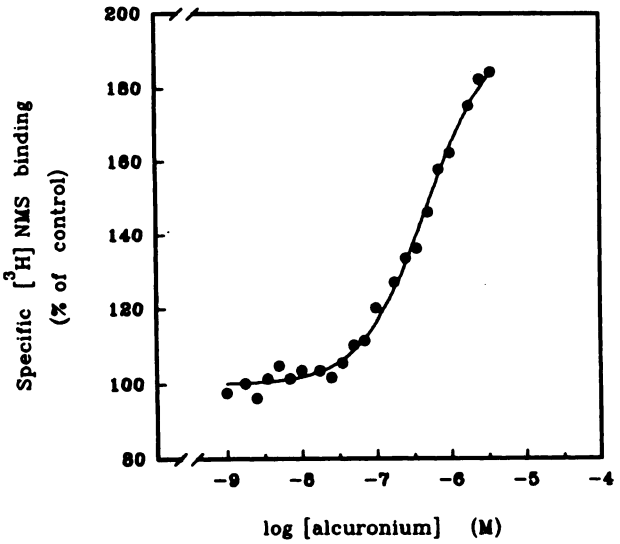

Fig. 7. Effect of increasing concentrations of alcuronium on the binding of [^{3}H]NMS after 5 hr of equilibration. Atrial membranes were incubated with alcuronium for 2 hr, after which [^{3}H]NMS was added to a concentration of 200 pM and the incubation was continued for an additional 5 hr. Data are means of three experiments, each performed with triplicate incubations. *Abscissa*, concentration of alcuronium; *ordinate*, binding of [^{3}H]NMS in the presence of alcuronium, expressed as percentage of binding in the absence of alcuronium. The *curve* has been drawn by computer, fitting data to an equation adapted from eq. 6 of Ehlert (4), i.e., $B_{[A]} = B_0 \times ([L] + K_{RL})/([L] + K'_{RL})$, where $B_{[A]}$ is the binding of [^{3}H]NMS in the presence of alcuronium, B_0 is the binding of [^{3}H]NMS in the absence of alcuronium, and $K'_{RL} = K_{RL} \times (K_{AR} + [A])/(K_{AR} + [A]/\alpha)$. Taking K_{RL} as 0.467 nM, the best fit was obtained for $K_{AR} = 667 \pm 34$ nM and $\alpha = 0.237 \pm 0.009$.

and the rate of [^{3}H]NMS dissociation from cardiac muscarinic receptors. (b) Alcuronium increases the binding of [^{3}H]NMS at subsaturating concentrations of the radioligand by increasing the affinity of receptors for [^{3}H]NMS; apparently, the ternary complex of alcuronium-[^{3}H]NMS-receptor is more stable thermodynamically than the binary complex of [^{3}H]NMS-receptor.

Two assumptions are involved in our interpretation of these findings, (a) that both alcuronium and [^{3}H]NMS bind directly to the receptor and (b) that alcuronium and [^{3}H]NMS do not compete for the same binding site, even at high concentrations of alcuronium. The first assumption is supported by the observations that the allosteric effects of gallamine are preserved after receptor solubilization (7) and that the same applies to the effects of alcuronium (25).

The second assumption is in fact one of the additional conclusions that we draw from the present experiments. In Fig. 5, the seeming inhibition of [^{3}H]NMS binding occurring at 3×10^{-5} M and 1×10^{-4} M alcuronium after 0.25 hr of incubation was transformed into a stimulation of radioligand binding after 3 or 20 hr of incubation. The extreme slowness with which [^{3}H]NMS associates with receptors in the presence of alcuronium (Fig. 1) is apparently responsible for the slowness of the equilibration of the system. In experiments shown in Figs. 3 and 4, receptors were preincubated with [^{3}H]NMS and 3×10^{-6} M alcuronium, thus reaching a high degree of occupancy with the radioligand, and subsequently incubated with concentrations of alcuronium of up to 3×10^{-4} M. In no case did the addition of more alcuronium diminish the amount of bound [^{3}H]NMS, as would be expected if the high concentrations of alcuronium competed for the [^{3}H]NMS binding sites; on the contrary, more [^{3}H]NMS binding occurred at increased alcuronium concentrations if sufficient time was given for additional equilibration (Fig. 5). Consequently, the inhibition of [^{3}H]NMS binding occurring in Fig. 5 at 1×10^{-3} M alcuronium is probably an artifact caused by the lack of time for equilibration, rather than the result of competition. Unfortunately, receptor molecules were not sufficiently stable for longer incubations under the conditions used.

If the binding of [^{3}H]NMS and alcuronium occurred to two distinct and sterically independent sites, it would be expected to proceed according to the scheme in Fig. 6a, obeying the law of mass action (4, 6) and leading to the formation of ARL complexes in two independent and reversible ways, via either RL or RA, where A is alcuronium, L is [^{3}H]NMS, and R is muscarinic receptor.

In our experiments, we have measured bound [^{3}H]NMS, which could have been in the form of both RL and ARL complexes, and we cannot distinguish between these two forms of bound radioligand. However, if the scheme in Fig. 6a reflects what is happening in our system, then an increase in the concentration of A should bring about an immediate increase in the rates of reactions 14 and 23 and, by causing an increase in the concentration of AR, also produce an increase in the rate of reaction 43. The rate of the formation of [RL + ARL] (i.e., the rate of the formation of bound [^{3}H]NMS) should be augmented when the concentration of A is raised, and data in Fig. 1 contradict this expectation.

There is only one way in which this anomalous behavior of the system may be explained; when a high concentration of alcuronium is added to the system, reaction 14 proceeds rapidly and most receptors bind alcuronium (high AR). The concentration of free receptors (R) becomes very low, and the rate of reaction 12 is slowed accordingly. That should be compensated for by an increase in the rate of reaction 43, because the concentration of the unlabeled AR complex is high. However, as Fig. 1 demonstrates, the higher the proportion of AR in the total concentration of R the slower is the combined rate of reactions 12 and 43 (i.e., the rate of [^{3}H]NMS binding), which can be explained only by concluding that reaction 43 does not proceed at all and that reaction 12 (plus subsequent reaction 23) is the only pathway permitting the formation of [^{3}H]NMS-receptor and [^{3}H]NMS-receptor-alcuronium complexes. In other words, [^{3}H]NMS cannot bind to the receptor after alcuronium, although alcuronium does increase the affinity of receptors towards [^{3}H]NMS (Figs. 1, 2, 4, 5, and 7; Table 3) and does slow down the dissociation of [^{3}H]NMS from receptors (Fig. 3; Table 2).

Similar restriction of reaction pathways apparently also applies to [^{3}H]NMS dissociation from the receptor. In the presence of alcuronium, the concentration of [^{3}H]NMS-receptor-alcuronium complexes is augmented. However, the higher the concentration of alcuronium the slower is [^{3}H]NMS dissociation observed after the addition of atropine (Fig. 2; Table 2). It should be noted that, after the addition of atropine, reactions 12 and 43 should be arrested but reactions 21 and 34 (i.e., net [^{3}H]NMS dissociation) should proceed unimpeded, at rates directly proportional to the concentrations of alcuronium-receptor-[^{3}H]NMS (ARL) and receptor-[^{3}H]NMS (RL) complexes. However, the more ARL there is in the system, the slower is the dissociation of L, and it is again necessary to conclude that reaction 34 does not proceed; i.e., that [^{3}H]NMS

cannot leave the receptor as long as alcuronium is attached. The only way for [^{3}H]NMS dissociation to occur is via reactions 32 and 21.

Apparently, [^{3}H]NMS can leave the receptor only during the short time intervals occurring between the spontaneous dissociation of one alcuronium molecule and the attachment of the next one. The higher the concentration of alcuronium, the shorter are such intervals and the less likely is the dissociation of [^{3}H]NMS. It seems likely that most [^{3}H]NMS is bound in ternary (ARL), rather than binary (RL), complexes with receptors in the presence of alcuronium (see Fig. 8 for the relevant model), because the affinity of receptors for [^{3}H]NMS is higher in these complexes. In accordance with this, [^{3}H]NMS appeared to dissociate from a single source at all concentrations of alcuronium higher than 3×10^{-6} M in experiments described in Fig. 3 and Table 2. The fast component of [^{3}H]NMS dissociation in experiments with 3×10^{-6} M alcuronium apparently reflected the presence of a proportion of receptors not associated with alcuronium.

It seems apparent that the chain of interactions between the receptor, alcuronium and [^{3}H]NMS is more correctly depicted by the scheme in Fig. 6b, than by that in Fig. 6a. To simplify further discussion and kinetic modeling, it proved useful to describe the four forms (one unliganded and three liganded) in which the receptor may be found by symbols R1–R4, as shown in the scheme in Fig. 6c.

Based on considerations explained in the Appendix and on kinetic constants derived from experimental data, we have constructed experimental models predicting the behavior of the system described in the schemes in Fig. 6, b and c, in three situations. (a) When fixed amounts of receptors and [^{3}H]NMS are incubated with increasing concentrations of alcuronium for increasing time periods (Fig. 5), a conspicuous feature of the model is the seeming 'inhibition' of [^{3}H]NMS binding at high concentrations of alcuronium, with a time-dependent shift of the descending part of the binding curve to the right. (b) When [^{3}H]NMS is added to a system in which receptors were pre-equilibrated with 3×10^{-6} M or 3×10^{-4} M alcuronium (Fig. 8), conspicuous features of the model are the slow equilibration (>50 hr at 3×10^{-4} M alcuronium) of [^{3}H]NMS binding and the high ratio between the concentrations of ARL and RL complexes. (c) When [^{3}H]NMS dissociation is elicited by the addition of excess atropine and its extent is followed in a system that was pre-equilibrated with [^{3}H]NMS and different concentrations of alcuronium (Fig. 9), conspicuous features of the model are the extreme slowness of dissociation at high concen-

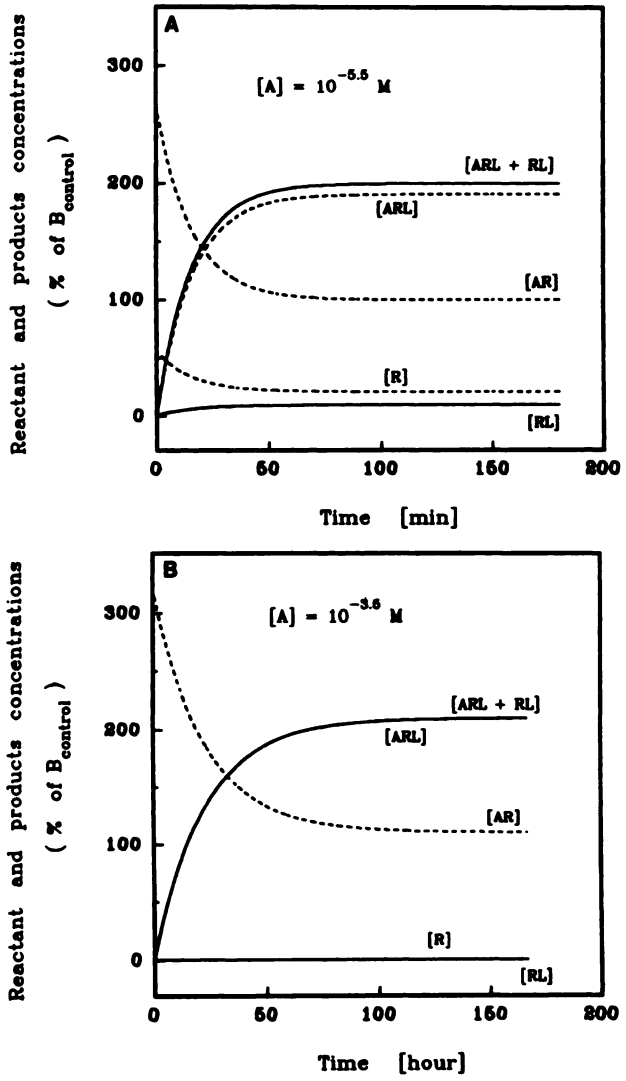

Fig. 8. Predicted time course of changes in the concentrations of reactants in a system corresponding to the scheme in Fig. 6b, after the addition of radiolabeled ligand L. The allosteric ligand A was equilibrated with the system before the addition of L; its concentration was 3×10^{-6} M (A) or 3×10^{-4} M (B). The *curves* were computed as described in Appendix C, based on the following parameters: [L] = 2×10^{-10} M, $[R_{TOT}] = 1.09 \times 10^{-12}$ M, $K_{AR} = 6.67 \times 10^{-7}$ M, $K_{ARL} = 1.58 \times 10^{-7}$ M, $k_{21} = 0.5$ min^{-1}, and $k_{12} = 1.14 \times 10^{9}$ min^{-1} M^{-1}. In B, the curves for [R] and [RL] are superimposed on each other, as are those for [ARL] and [ARL + RL]. *Ordinate*, percentage of the concentration of RL in the system that was equilibrated with L in the absence of A. Note that, in the context of the present study, A corresponds to alcuronium, L to [^{3}H] NMS, and the sum [RL + ARL] to bound radioactivity.

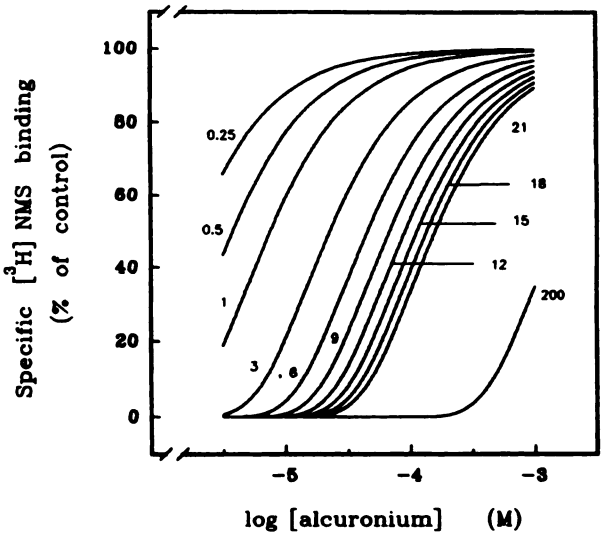

Fig. 9. Predicted effects of increasing concentrations of alcuronium on the dissociation of [^{3}H]NMS from receptors. The system was equilibrated with alcuronium and [^{3}H]NMS and dissociation was started by the addition of atropine. *Abscissa*, concentration of alcuronium; *ordinate*, [^{3}H]NMS binding after *n* hours of dissociation (the duration of dissociation, in hours, is indicated by *numbers at each curve*), expressed as percentage of [^{3}H]NMS binding before the addition of atropine. Based on the integrated form of eq. 9 (Appendix B), the curves have been drawn to obey the equation $[\% B_{conc}]_t = 100 \exp\{-(K_{ARL}/[A]) \cdot k_{21} t\}$, taking K_{ARL} = 158 nM and $k_{21} = 0.5$ min^{-1}.

trations of alcuronium and the seeming 'biphasicity' of the effects of alcuronium on dissociation.

To summarize, we propose that alcuronium has two effects on cardiac muscarinic receptors. (a) It allosterically increases the affinity of receptors for NMS, presumably by inducing a conformational change in the immediate vicinity of the binding site for NMS. (b) It blocks the pathway for the access of NMS to its binding site and for the departure of NMS from the binding site. Because the binding site for NMS is located in a hydrophobic pocket at the level of the membrane (26), one can imagine either that alcuronium obstructs the passage (tunnel?) to the binding site by directly binding at its entrance or in its course or that it binds at a distance and induces a change of protein conformation that blocks the access to the binding site. The view that alcuronium acts by direct steric hindrance is supported by experiments performed in our laboratory,[1] indicating that alcuronium protects the muscarinic orthosteric binding site against inactivation by several protein-modifying reagents. Steric obstruction thus appears a likely mechanism for the effects of alcuronium on [^{3}H]NMS association and dissociation rates. Steric effects similar to those that we postulate for alcuronium have been described in enzymological literature (27). A simplified view of how alcuronium and NMS associate with the receptor in four basic situations envisioned in the scheme in Fig. 6c is provided in Fig. 10.

Acknowledgments

We thank Professor H. Bachelard, Dr. A. Kaiser, Mr. P. Weber, and The Wellcome Trust for assistance in providing reagents.

Appendix

A. Derivation of apparent monomolecular rate constants for reactions in the scheme in Fig. 6b. The following conditions have been fulfilled in all experiments:

$$[L[\gg [R]$$

$$[A] \gg [R]$$

All reaction steps in the scheme in Fig. 6b can therefore be treated as pseudomonomolecular reactions, which permits the use of the formalized description applied in Fig. 6c and simplifies subsequent treatment.

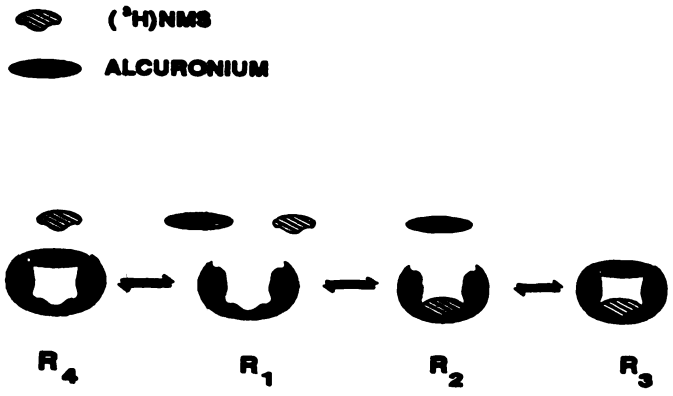

Fig. 10. Schematic drawing of proposed interactions between alcuronium, NMS, and muscarinic receptors. Receptor forms R_1–R_4 correspond to those in the scheme in Fig. 6c.

[1] J. Jakubík and S. Tuček. Protection by alcuronium of muscarinic receptors against chemical inactivation and location of the allosteric binding site for alcuronium. Manuscript in preparation.

The rate of individual reaction steps may be described by the equations

$$v_{12} = k_{12}[L][R] = \omega_{12}[R] = \omega_{12}[R_1]$$

$$v_{41} = k_{41}[AR] = \omega_{41}[AR] = \omega_{41}[R_4]$$

$$v_{23} = k_{23}[RL][A] = \omega_{23}[RL] = \omega_{23}[R_2]$$

$$v_{21} = k_{21}[RL] = \omega_{21}[RL] = \omega_{21}[R_2]$$

$$v_{14} = k_{14}[A][R] = \omega_{14}[R] = \omega_{14}[R_1]$$

$$v_{32} = k_{32}[ARL] = \omega_{32}[ARL] = \omega_{32}[R_3]$$

so that the apparent rate constants ω are equal to

$$\omega_{21} = k_{21}$$

$$\omega_{32} = k_{32}$$

$$\omega_{41} = k_{41}$$

$$\omega_{12} = k_{12}[L]$$

$$\omega_{23} = k_{23}[A]$$

$$\omega_{14} = k_{14}[A]$$

B. Derivation of the equilibrium dissociation constant of the complex ARL, based on the kinetics of dissociation in the presence of excess atropine. It follows from the scheme in Fig. 6b that the addition of excess atropine, preventing the reassociation of [^{3}H]NMS, is followed by

$$ARL \underset{23}{\overset{32}{\rightleftarrows}} A + RL \xrightarrow{21} A + R + L$$

which may be expressed according to Fig. 6c

$$R_3 \underset{23}{\overset{32}{\rightleftarrows}} R_2$$

$$R_2 \xrightarrow{21} R_1$$

Using the pseudomonomolecular rate constants derived in A, we obtain a system of three differential equations

$$\frac{d[R_3]}{dt} = -\omega_{32}[R_3] + \omega_{23}[R_2] \quad (1)$$

$$\frac{d[R_2]}{dt} = \omega_{32}[R_3] - \omega_{23}[R_2] - \omega_{21}[R_2] \quad (2)$$

$$\frac{d[R_1]}{dt} = \omega_{21}[R_2] \quad (3)$$

The condition has been fulfilled in our system that $[A] \gg [L] \gg [R_2]$. Under such circumstances, R_2 behaves as an unstable intermediate, and

$$\frac{d[R_2]}{dt} = 0 \quad (4)$$

After combining eqs. 2 and 4 and rearranging, we obtain

$$[R_2] = \frac{\omega_{32}}{\omega_{23} + \omega_{21}}[R_3] \quad (5)$$

Under the conditions defined, the relation holds that $\omega_{23} \gg \omega_{21}$, and one can write with sufficient precision

$$[R_2] = \frac{\omega_{32}}{\omega_{23}}[R_3] \quad (6)$$

If we express ω_{23} and ω_{32} according to definitions in A, eq. 6 is transformed to

$$[R_2] = \frac{k_{32}}{k_{23}[A]}[R_3] = \frac{K_{ARL}}{[A]}[R_3] \quad (7)$$

where K_{ARL} is the dissociation constant of the complex ARL for the reaction

$$ARL \rightleftarrows A + RL$$

It follows from eq. 4 that, for the present system,

$$\frac{d[R_3]}{dt} = -\frac{d[R_1]}{dt} = -\omega_{21}[R_2] \quad (8)$$

Substituting from eq. 7 to eq. 8, and because $\omega_{21} = k_{21}$, we can write

$$\frac{d[R_3]}{dt} = -k_{21}\frac{K_{ARL}}{[A]}[R_3] = -{}^{[A]}k_{off}[R_3] \quad (9)$$

where ${}^{[A]}k_{off}$ corresponds to the observed rate constant of the dissociation of radiolabel from receptors. The superscript [A] indicates the respective concentration of alcuronium. The value of ${}^{[A]}k_{off}$ can be determined experimentally and its observed values have been listed in Table 2 as $k^*_{off_1}$.

For K_{ARL} we can write

$$K_{ARL} = \frac{{}^{[A]}k_{off}}{k_{21}}[A] \quad (10)$$

Values of K_{ARL} calculated from observed $k^*_{off_1}$ values according to eq. 10 have been listed in Table 4. One may note their close correspondence to the value of 158 nM obtained in Results from the estimates of K_{AR} and α.

C. Modeling of the kinetic behavior of the system in the scheme in Fig. 6c. For modeling of the kinetic behavior of the system described in the scheme in Fig. 6c, one has to resolve the following homogeneous system of linear differential equations:

$$\frac{d[R_1]}{dt} = -(\omega_{12} + \omega_{14})[R_1] + \omega_{21}[R_2] + \omega_{41}[R_4]$$

$$\frac{d[R_2]}{dt} = \omega_{12}[R_1] - (\omega_{21} + \omega_{23})[R_2] + \omega_{32}[R_3]$$

$$\frac{d[R_3]}{dt} = \omega_{23}[R_2] - \omega_{32}[R_3]$$

$$\frac{d[R_4]}{dt} = \omega_{14}[R_1] - \omega_{41}[R_4]$$

Although it is still possible to obtain analytical solutions for R_1, R_2, R_3, and R_4, the extreme complexity of the obtained expressions makes it necessary to use numerical methods. We have used the KINSIM program (28) for simulation of the kinetic behavior of the proposed model. The values of k_{12} and k_{11} have been derived from experiments on [^{3}H]NMS association and dissociation (Figs. 1 and 3) and K_{AR} and K_{ARL} from experiments measuring the positive effect of alcuronium on [^{3}H]NMS binding at equilibrium (Fig. 7).

TABLE 4

Calculated values of K_{ARL}

The calculation was performed according to eq. 10, based on data in Table 2

Alcuronium concentration	K_{ARL}
M	nM
3×10^{-6}	128
1×10^{-5}	140
3×10^{-5}	176
1×10^{-4}	218
3×10^{-4}	189

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