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Competition between Positive and Negative Allosteric Effectors on Muscarinic Receptors

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SUMMARY

Alcuronium allosterically increases the affinity of cardiac muscarinic receptors for methyl-*N*-scopolamine (NMS), whereas gallamine has the opposite effect. We discovered that strychnine also increases the affinity of muscarinic receptors in rat heart atria for NMS. It is not known whether the positive and the negative allosteric effectors bind to the same binding site. To investigate this question, we elaborated on a theoretical model predicting changes in the binding of a classic radiolabeled ligand occurring in the presence of a positive and a negative allosteric effector that compete for the allosteric binding site. The model is based on data obtained at equilibrium and avoids uncertainties associated with the use of nonequilibrium methods for the evaluation of interactions between allosteric ligands. We examined changes in the binding of [^{3}H]NMS to membranes of rat heart atria exposed to various concentrations of a positive allosteric effector (alcuronium or strychnine) and of a negative allosteric effector (gallamine) simultaneously. The binding data obtained were in perfect agreement with the model assuming competition between gallamine and alcuronium and gallamine and strychnine, strongly suggesting that these positive and negative allosteric effectors bind to identical or overlapping sites.

Many substances have allosteric effects on muscarinic receptors, but little is known about the structure, role, and properties of the site(s) with which they associate (see reviews in Refs. 1–3). It is not clear whether there is just one allosteric binding site for the various allosteric ligands or whether these sites are multiple and how specific they are regarding their potential ligands. Progress in the field is hampered by the unavailability of radiolabeled ligands specific for allosteric sites and by the dearth of theoretical models with which to analyze relations between unlabeled ligands binding to the allosteric site(s). The need for such models has been demonstrated recently (4–7).

The most thoroughly studied allosteric ligand at muscarinic receptors has been the neuromuscular blocking drug gallamine (8–12), which has a high affinity for the receptors of the M_2 subtype and decreases their affinity for all classic muscarinic ligands that have been investigated. We have shown that alcuronium, another neuromuscular blocker, has a positive allosteric effect on the binding of NMS to M_2 receptors, whereas it has a negative effect on the binding of quinuclidinyl benzilate (13–15); positive and negative interactions were also discovered between alcuronium and other ligands associating with the classic site on muscarinic receptors (16). In the present report, we report that strychnine (the molecule of which has substantial similarities with alcuronium) also displays a positive allosteric action on the binding of NMS. We were concerned with whether the positive and the negative allosteric effectors, as exemplified by alcuronium, strychnine, and gallamine (Fig. 1), bind to the same binding site, for which they compete, or to different sites.

To investigate the relation among the binding of alcuronium, strychnine, and gallamine, we measured the equilibrium binding of [^{3}H]NMS to cardiac muscarinic receptors in the presence of various concentrations of alcuronium, strychnine, and gallamine added either separately or in combination and compared the data obtained with what would be expected according to a theoretical model for competition between the allosteric effectors, based on data obtained at equilibrium and on the analysis of allosteric binding provided by Ehlert (17) and Proška and Tuček (15). We tried to examine the relations between the binding of allosteric effectors without the use of parameters derived from measurements of the rates of the dissociation of radiolabeled ligands (5, 6) because theoretically these parameters appear to be controversial, as we point out in Discussion.

Materials and Methods

Experiments were performed on homogenates of rat heart atria as described (15), with minor modifications. Tissue was homogenized with a polytron-type homogenizer, and the homogenization and in-

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ABBREVIATION: NMS, methyl-*N*-scopolamine.

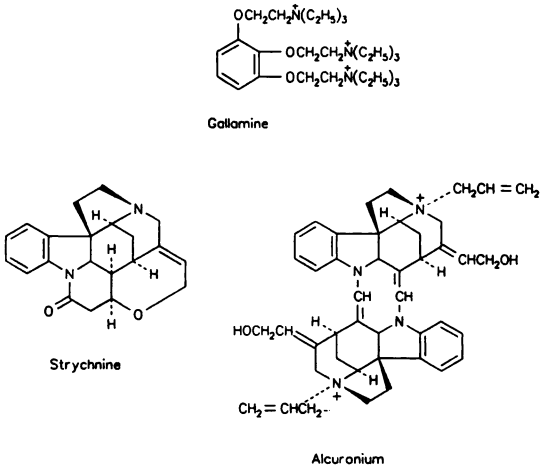

Fig. 1. Structures of gallamine, strychnine, and alcuronium.

cubation medium consisted of 100 mM NaCl and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; the concentration of [^{3}H]NMS applied throughout was 200 pM, close to half of the K_d for [^{3}H]NMS (416 pM under the conditions used). The incubation was at 25° in a volume of 1.6 ml and lasted 5 hr unless indicated otherwise. Whatman GF/B glass fiber filters (presoaked in 0.1% polyethylenimine for 3 hr) were used for the separation of the bound radioligand. Alcuronium was kindly provided by Hoffmann-La Roche (Basel), and strychnine hemisulfate was from Sigma Chemical Co. (St. Louis, MO).

Data treatment and modeling were performed as described (15). Symbols are applied in their generally accepted meaning. To quantify the cooperativity between the binding of [^{3}H]NMS and the allosteric effectors, cooperativity factors were computed according to Ehlert (17) and denoted by α in the case of cooperativity between the binding of [^{3}H]NMS and the positive allosteric effectors (i.e., alcuronium or strychnine) and by β in the case of cooperativity between the binding of [^{3}H]NMS and the negative allosteric effector (gallamine). An L is used to denote the classic labeled ligand (i.e., [^{3}H]NMS in our experiments), whereas A indicates the allosteric ligand with a positive effect, and G indicates the allosteric ligand with a negative effect on the binding of L.

Results

[^{3}H]NMS binding in the presence of alcuronium, strychnine, or gallamine applied alone. Pilot experiments that we performed with various substances indicated that strychnine has a positive allosteric effect on the binding of [^{3}H]NMS to cardiac muscarinic receptors, similar to that of alcuronium. The effect of increasing concentrations of strychnine on the binding of [^{3}H]NMS to membranes of rat heart atria is shown in Fig. 2 after 2- and 5-hr incubations. It can be seen that 10 μM strychnine increased the binding of [^{3}H]NMS (which was present at a concentration corresponding to approximately half of its K_d value) by 60% and that a prolongation of incubation from 2 to 5 hr did not change the binding of [^{3}H]NMS at strychnine concentrations of <10 μM, whereas it increased [^{3}H]NMS binding at higher concentrations, apparently (by analogy with the effects of alcuronium; see Ref. 15) by providing more time for the decelerated radioligand association.

The effects of increasing concentrations of alcuronium, strychnine, and gallamine on the binding of [^{3}H]NMS during 5-hr incubation have been compared in Fig. 3; in these and subsequent experiments, the value of K_{RL} (K_d for [^{3}H]NMS)

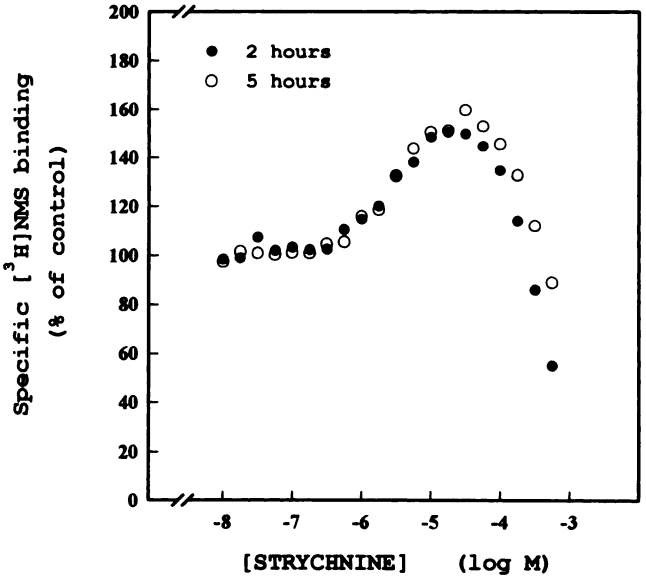

Fig. 2. Changes in the specific binding of [^{3}H]NMS to cardiac muscarinic receptors in the presence of increasing concentrations of strychnine during incubations for 2 or 5 hr. *Abscissa*, log molar concentration of strychnine hemisulfate. *Ordinate*, percentage of binding in the absence of strychnine. For each incubation, values are mean of four experiments with incubations performed in duplicates.

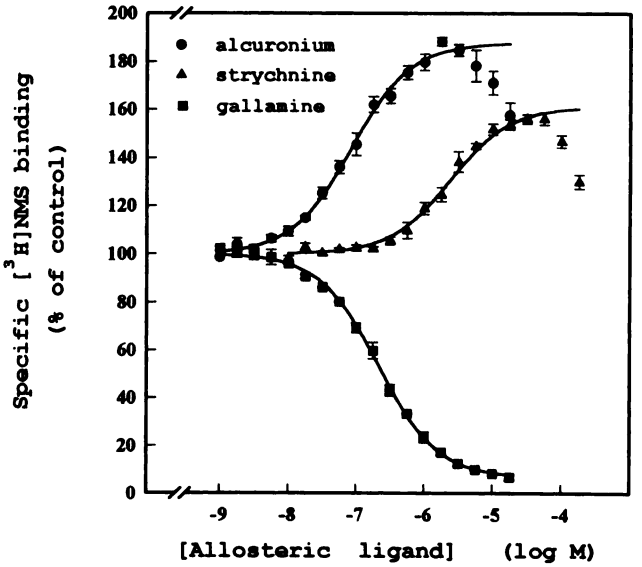

Fig. 3. The effect of increasing concentrations of allosteric effectors alcuronium, strychnine, and gallamine on the binding of [^{3}H]NMS to muscarinic receptors in rat heart atria. *Abscissa*, log molar concentration of allosteric effectors. *Ordinate*, percentage of binding in the absence of allosteric effectors. The concentration of [^{3}H]NMS was 200 pM, and the incubation was at 25° and lasted 5 hr. Values are mean ± standard error of four experiments with incubation in duplicates. Full curves represent the best fit of eq. 6 from Ehlert (17) to experimental data within the concentration range of 10^{-9} to $10^{-5.5}$ M alcuronium and gallamine and 10^{-8} to $10^{-4.5}$ M strychnine, for K_{RL} = 0.416 nM, K_{AR} = 146 nM for alcuronium, K_{AR} = 3364 nM for strychnine, K_{GR} = 149 nM for gallamine, and cooperativity coefficients α = 0.31 for alcuronium, α = 0.44 for strychnine, and β = 21.37 for gallamine.

was 0.416 nM. Fitting Eq. 6 from the work of Ehlert (17) to the data obtained at alcuronium and gallamine concentrations in the range of 10^{-9} to $10^{-5.5}$ M and at strychnine concentrations in the range of 10^{-8} to $10^{-4.5}$ M yielded K_{AR} = 146 nM and α = 0.31 for alcuronium, K_{AR} = 3364 nM and α =

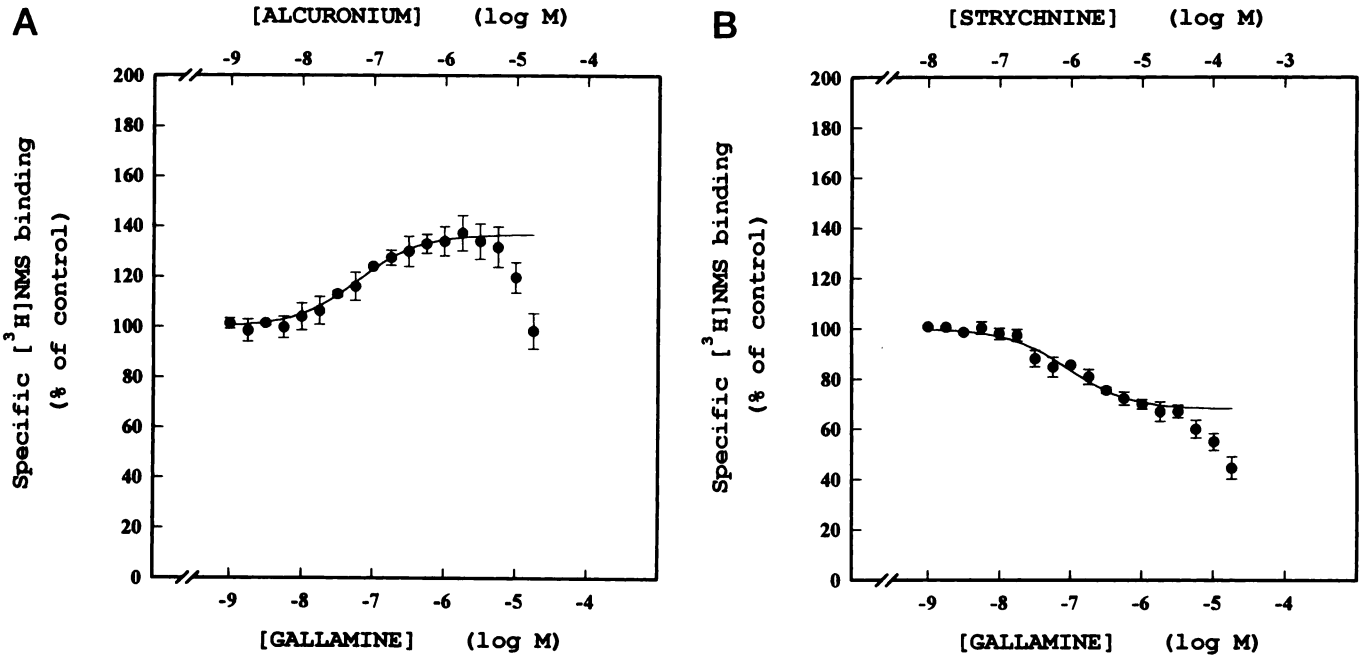

Fig. 4. A, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and alcuronium. B, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and strychnine. *Abscissa*, log molar concentration of gallamine (*bottom*) and of alcuronium or strychnine (*top*). *Ordinate*, percentage of binding in the absence of allosteric effectors. Values are mean ± standard error of four experiments with incubation in duplicates. Full lines are computer-generated curves (not best fits to data) corresponding to eq. 13 and to parameters obtained in separate independent experiments and described in the legend to Fig. 3.

0.44 for strychnine, and K_{GR} = 149 nM and β = 21.37 for gallamine. Within the 5-hr incubation period used, the binding of [^{3}H]NMS did not reach equilibrium in the presence of gallamine or alcuronium at concentrations of $>10^{-5.5}$ M or of strychnine at concentrations of $>10^{-4.5}$ M.

[^{3}H]NMS binding in the simultaneous presence of alcuronium and gallamine or of strychnine and gallamine. Figs. 4 and 5 show data obtained in three differently arranged experiments in which alcuronium and gallamine or strychnine and gallamine were allowed to bind to receptors simultaneously, presumably competing for the allosteric binding sites and influencing the binding of [^{3}H]NMS to the orthosteric sites in both a positive (alcuronium or strychnine) and a negative (gallamine) direction. In Fig. 4, the concentrations of the positive and the negative allosteric effector were increased in parallel. It can be seen that the positive effects of alcuronium and strychnine and the negative effect of gallamine on [^{3}H]NMS binding in large part neutralized each other, with a slight prevalence of the positive action of alcuronium over gallamine and of gallamine over strychnine; the apparent strong inhibition of [^{3}H]NMS binding at 10^{-5} M gallamine and 10^{-4} M strychnine in Fig. 4B is likely to be due to insufficient equilibration.

In Fig. 5, A and B, the concentrations of alcuronium and strychnine were gradually diminished, whereas the concentration of (simultaneously present) gallamine was augmented. This arrangement resulted in steep binding curves, showing transition from stimulation to inhibition of binding. In Fig. 5, C and D, incubations were in the presence of increasing concentrations of gallamine and of fixed concentrations of alcuronium ($10^{-5.5}$ or $10^{-6.5}$ M) or strychnine ($10^{-4.5}$ or $10^{-5.5}$ M). Again, the curves showed a conspicuous transition from the stimulation to the inhibition of binding, with characteristic rightward shifts of the binding curves when the concentrations of alcuronium (Fig. 5C) or strychnine (Fig. 5D) were augmented by 1 order of magnitude.

Theoretical model. Events occurring in the system have been schematically described in Fig. 6. It has been shown (15) that the orthosteric binding site is not accessible to [^{3}H]NMS as long as the allosteric site is occupied by alcuronium, and similarities between alcuronium and gallamine in their actions on the rates of [^{3}H]NMS association and dissociaton support the assumption that gallamine hinders the access to the binding site for [^{3}H]NMS in the same way as does alcuronium (18). Two reactions were therefore crossed out in what otherwise would be a bicyclic binding scheme. It should be noted, however, that the system that we used for measurements was at equilibrium and therefore independent of the pathways by which the equilibrium state had been reached.

For the situation in Fig. 6, we can write:

$$K_{RL} = \frac{[L]\,[R]}{[RL]} \quad (1)$$

$$K_{AR} = \frac{[A]\,[R]}{[AR]} \quad (2)$$

$$K_{GR} = \frac{[G]\,[R]}{[GR]} \quad (3)$$

$$\alpha K_{AR} = \frac{[A]\,[RL]}{[ARL]} \quad (4)$$

$$\beta K_{GR} = \frac{[G]\,[RL]}{[GRL]} \quad (5)$$

$$[R_{TOT}] = [RL] + [AR] + [GR] + [ARL] + [GRL] + [R] \quad (6)$$

In this scheme, L denotes labeled classic ligand [^{3}H]NMS, whereas A denotes positive allosteric effectors alcuronium and strychnine, and G denotes the negative allosteric effector gallamine. The α and β denote cooperativity factors for the interactions between A and L or G and L, respectively. The following derivation extends the basic ideas of Ehlert (17) to a more complex system.

Taking Y = [RL] + [ARL] + [GRL], the fraction of the labeled forms of the receptor is as follows:

$$\frac{Y}{[R_{TOT}]} = \frac{[RL] + [ARL] + [GRL]}{[RL] + [AR] + [GR] + [ARL] + [GRL] + [R]} \quad (7)$$

Substituting from eq. 1–5 to eq. 7 gives the following:

$$\frac{Y}{[R_{TOT}]} = \frac{\frac{1}{K_{RL}}\left(1 + \frac{[A]}{\alpha K_{AR}} + \frac{[G]}{\beta K_{GR}}\right)}{\frac{1}{K_{RL}} + \frac{[A]}{K_{AR}[L]} + \frac{[G]}{K_{GR}[L]} + \frac{[A]}{\alpha K_{AR} K_{RL}} + \frac{[G]}{\beta K_{GR} K_{RL}} + \frac{1}{[L]}} \quad (8)$$

Eliminating the fractions in the numerator and denominator, inserting normalized concentrations a = [A]/K_{AR} and g = [G]/K_{GR}, and simplifying yields the following:

$$\frac{Y}{[R_{TOT}]} = \frac{[L]}{[L] + K_{RL}\left(\frac{a + g + 1}{a/\alpha + g/\beta + 1}\right)} \quad (9)$$

Applying a substitution in eq. 9 yields the following:

$$Y = \frac{[L]\,[R_{TOT}]}{[L] + (K_{RL})_{A,G}} \quad (10)$$

where

$$(K_{RL})_{A,G} = K_{RL}\left(\frac{a + g + 1}{a/\alpha + g/\beta + 1}\right) \quad (11)$$

equals apparent K_{RL} in the presence of A and G. The fractional binding of L in the presence of A and G is described by the following expression:

$$Y/Y_o = \frac{[L] + K_{RL}}{[L] + (K_{RL})_{A,G}} \quad (12)$$

where Y_o is the binding of L in the absence of A and G.

Finally, we obtain the following expression

$$\%B_{control} = 100\left[\frac{[L] + K_{RL}}{[L] + K_{RL}\left(\frac{a + g + 1}{a/\alpha + g/\beta + 1}\right)}\right] \quad (13)$$

where $B_{control}$ represents the binding of L in the absence of A and G.

Note that all parameters used in eq. 13 can be derived from equilibrium binding experiments, i.e., from saturation binding experiments with [^{3}H]NMS (K_{RL}) and from experiments of the type shown in Fig. 3 for alcuronium and strychnine (α; K_{AR} for the calculation of a) and for gallamine (β; K_{GR} for the calculation of g).

Comparison of model and experimental data. Full lines in Figs. 4 and 5 represent the expected values computed according to eq. 13 for the binding of [^{3}H]NMS at different concentrations of alcuronium, strychnine, and gallamine, assuming that K_{LR} = 0.416 nM, K_{AR} = 146 nM and α = 0.31 for alcuronium, K_{AR} = 3364 nM and α = 0.44 for strychnine, and K_{GR} = 149 nM and β = 21.37 for gallamine (see Fig. 3). Perfect fit between the results of actual measurements and the model curves is easy to notice; discordance appears only at high concentrations of allosteric ligands because of the extremely slow equilibration (15).

Discussion

Although the original observation that alcuronium has a tissue-selective positive allosteric effect on the binding of [^{3}H]NMS to muscarinic receptors was reported 8 years ago (13), no other substances have been found that have a similar action. There is no doubt, however, that drugs acting as subtype-selective allosteric enhancers of classic muscarinic ligands could be extremely useful. The finding that strychnine is also able to enhance the binding of [^{3}H]NMS to cardiac receptors indicates that alcuronium is not unique in its position of a positive allosteric effector on muscarinic receptors and is likely to be of use in the search for substances with higher potencies and efficacies and in the elucidation of structural requirements that the positive allosteric effectors must fulfill. The action of strychnine is not surprising because its chemical structure is similar to that of alcuronium and the substance is used as the starting material for the chemical synthesis of alcuronium (19, 20). With regard to neurotransmitter receptors, however, strychnine has been known only as a specific and probably allosteric antagonist of ionotropic receptors for glycine (21–23), whereas indications of its potential relation to the receptors for acetylcholine were sporadic (24–26).

The number of compounds with allosteric effects on muscarinic receptors is large (1, 3, 27, 28), and it is a question of principal importance whether they all bind to the same allosteric binding site or whether there are multiple allosteric binding sites on muscarinic receptors. Competition for binding sites should be apparent in the case of simultaneous binding to the same binding site, and the availability of drugs that have opposite (positive and negative) effects on the affinity of a receptor for its classic ligands should facilitate the investigation of such competition.

The model that we propose for the evaluation of competitive interactions between the positive and the negative allosteric effectors relies on the measurements of the changes in the binding of a radiolabeled classic antagonist that are induced by unlabeled allosteric effectors and thus overcomes the difficulty stemming from the fact that suitable radiolabeled allosteric effectors are not available. The model extends the basic ideas of Ehlert (17) and the approaches applied in previous work (15) and is completely based on measurements performed at binding equilibrium. In this way, it avoids complications arising from uncertainties in the understanding of individual kinetic steps through which the equilibrium is achieved.

We measured changes in the binding of [^{3}H]NMS occurring in the presence of gallamine and either alcuronium or strychnine under conditions when the concentrations of both the negative and the positive effector were increasing, when the concentrations were changing in opposite directions, or when the concentrations of the positive effectors were fixed while

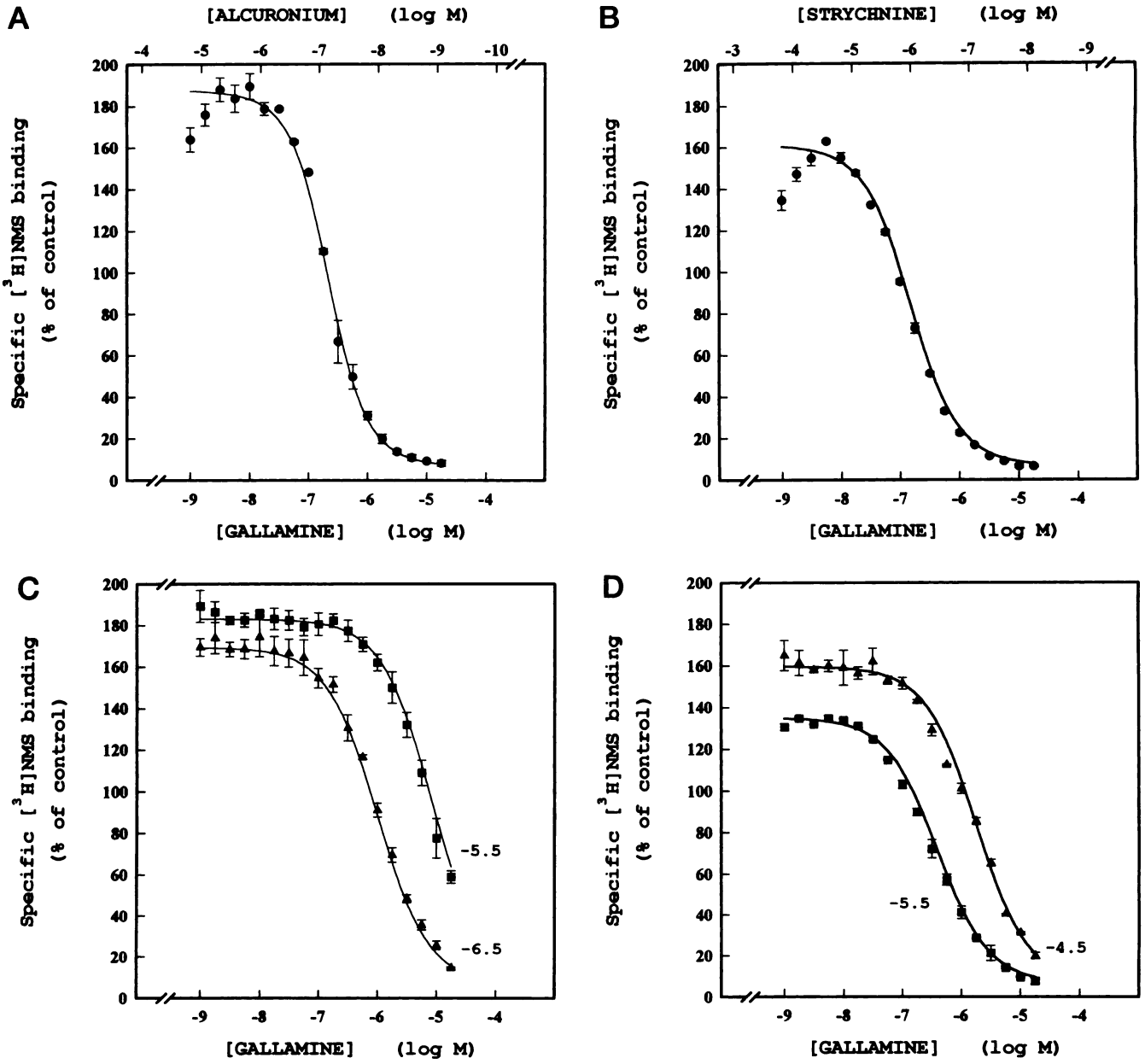

Fig. 5. A, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and decreasing concentrations of alcuronium. B, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and decreasing concentrations of strychnine. C, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and two fixed concentrations of alcuronium ($10^{-6.5}$ and $10^{-5.5}$ M). D, Changes in the binding of [^{3}H]NMS in the simultaneous presence of increasing concentrations of gallamine and two fixed concentrations of strychnine ($10^{-5.5}$ and $10^{-4.5}$ M). *Abscissa*, log molar concentration of gallamine (*bottom*) and of alcuronium or strychnine (*top*, A and B). *Ordinate*, percentage of binding in the absence of allosteric effectors. Values are mean ± standard error of four experiments with incubation in duplicates. Full lines are computer-generated curves (not best fits to data) corresponding to eq. 13 and to parameters obtained in separate independent experiments and described in the legend to Fig. 3.

those of the negative effector varied. Under all circumstances, perfect fit has been obtained between what had been predicted by the model and what was found experimentally. It should be stressed that the parameters used for drawing the curves predicted by the model were derived from separate, independent experiments and were not obtained by fitting the model (i.e., eq. 13) to experimental data.

Based on the full agreement between the model and the experimental data, it appears justified to conclude that alcuronium and gallamine, and strychnine and gallamine compete for binding to the receptor. Their binding sites are probably localized in the same receptor domain and are either identical or overlapping. This is important for at least two reasons: (a) it indicates that both positive and negative allosteric effects can be evoked from the same receptor domain, and (b) it indicates that the same domain is probably responsible for the binding of multiple allosteric effectors with chemical structures that can be as different as are those of gallamine and alcuronium. Investigations of the effects of alcuronium on the kinetics of receptor interaction with [^{3}H]NMS led to the conclusion that the site to which alcuronium binds is probably located near the entry to the pocket in

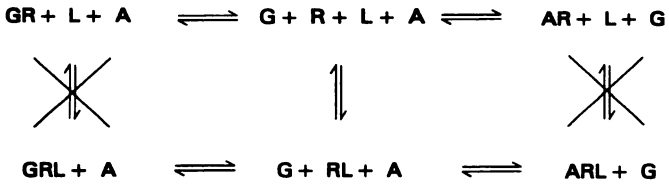

Fig. 6. Interactions between receptor R, classic ligand L ([^{3}H]NMS in the present study), and allosteric ligands A (alcuronium or strychnine) and G (gallamine). *Crossed reactions* do not proceed because the binding of the allosteric ligands presumably creates a steric obstacle hindering the movement of L (15).

which the classic binding site is located (15), and support for this view has been obtained in experiments with chemical modifications of the receptor (29, 30) and, with regard to the binding of gallamine, in experiments with site-directed mutagenesis (18).

Recently, studies have indicated that gallamine and obidoxime (5) and gallamine and *d*-tubocurarine (6) compete for their binding sites on muscarinic receptors. In the former study, this conclusion was drawn from an investigation of the effects of allosteric modulators on the rates of atropine-induced [^{3}H]NMS dissociation. Such an approach is based on two assumptions that deserve comment.

One assumption (a) is that the dissociation of L from receptors occupied by the allosteric ligand (A or G) induced by a high concentration of atropine or another classic antagonist is a monomolecular unidirectional process that apparently follows the equation ARL → AR + L or GRL → GR + L and therefore depends on the concentration and properties of ARL or GRL only. Very probably, however, the dissociation of [^{3}H]NMS can proceed only after the dissociation of A or G (Fig. 6; Refs. 15 and 18), so the likely sequence of events is ARL ⇌ A + RL → A + R + L ⇌ AR + L or GRL ⇌ G + RL → G + R + L ⇌ GR + L (atropine is omitted from the equations for simplification), and the concentrations of free A and free G in the medium consequently become of importance for the rate of dissociation. The higher [A] or [G], the more likely it is that the reassociation of RL with A or G to ARL or GRL occurs faster than the dissociation of RL to R + L, so L remains "trapped" in the ternary complex.

Another assumption (b) is that the rate of dissociation is proportional to receptor occupancy by A or G. Experimental data supporting the validity of this assumption have not been published. We have observed that the rate of [^{3}H]NMS dissociation was >20-fold diminished when the concentration of alcuronium rose from 10 μM to 300 μM, although receptor occupancy by alcuronium was close to 100% within this concentration range (see Table 2 in Ref. 15). Data in Fig. 7 show the results of experiments that we performed within the framework of the present study. In these experiments, the dissociation of [^{3}H]NMS from cardiac receptors was induced by the addition of gallamine (alone, without atropine). It can be seen from Fig. 7 that an increase in the concentration of gallamine from 10^{-4} M to 10^{-3} M is associated with a pronounced deceleration of [^{3}H]NMS dissociation, although, based on data from measurements at equilibrium, the K_d of gallamine for its binding to the receptor occupied by [^{3}H]NMS (computed as $\beta \times K_{GR}$) is as low as 3.2×10^{-6} M. The observed effects of suprasaturating concentrations of gallamine and alcuronium agree with what was deduced in the previous paragraph.

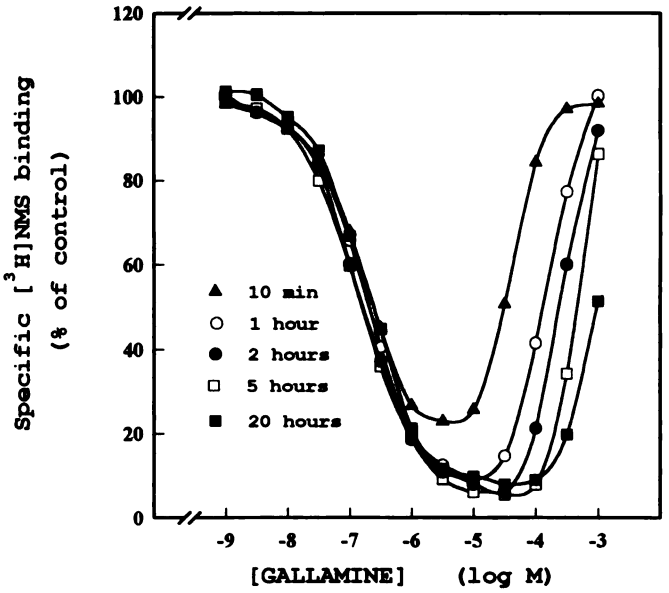

Fig. 7. Gallamine-induced dissociation of [^{3}H]NMS from cardiac muscarinic receptors showing its dependence on the concentration of gallamine and on time. Rat atrial membranes were preincubated with 200 pM [^{3}H]NMS for 2 hr, and then gallamine was added to final concentrations of 10^{-9} to 10^{-3} M (abscissa). The incubation was terminated by filtration 10 min to 20 hr after the addition of gallamine; symbols for each duration of incubation are shown in the graph. *Abscissa*, log molar concentration of gallamine. *Ordinate*, binding of [^{3}H]NMS revealed at the indicated time interval and at the indicated concentration of gallamine, expressed as percentage of binding in parallel samples to which no gallamine had been added. Each point is the mean of four experiments. Full lines were obtained by polynomial interpolation. More than six experiments performed with a different incubation medium gave closely similar results.

In conclusion, we presented a model that can be used to predict changes in the binding of a labeled classic ligand when the binding is affected by a positive and a negative allosteric effector that bind simultaneously and compete between themselves for the allosteric binding site. We discovered that strychnine has a positive allosteric effect on the binding of [^{3}H]NMS to cardiac muscarinic receptors and have shown that the negative allosteric effector gallamine apparently competes for the allosteric binding site on cardiac muscarinic receptors with the positive allosteric effectors alcuronium and strychnine.

Acknowledgments

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