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ACCELERATED COMMUNICATION

Gallamine Exerts Biphasic Allosteric Effects at Muscarinic Receptors

JOHN ELLIS and MARGARET SEIDENBERG

Neuroscience Research Unit, Department of Psychiatry, University of Vermont College of Medicine, Burlington, Vermont 05405

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SUMMARY

Although gallamine and a number of other compounds have been reported to slow the rate of dissociation of labeled ligands, especially [^{3}H]*N*-methylscopolamine (NMS), from muscarinic receptors of heart and brain, there has been some dispute as to whether the dissociation of [^{3}H]quinuclidinyl benzilate (QNB) is subject to such allosteric regulation. The present studies were intended to determine whether past discrepancies might be due to differences between tissues. We have found that gallamine modulates the dissociation of [^{3}H]QNB from muscarinic receptors of the heart in a biphasic manner. Low concentrations (micromolar) accelerate the rate of dissociation, whereas higher concentrations (millimolar) slow it; at about 0.1 mM, the two effects cancel each other. Similar results were obtained with muscarinic receptors from the brainstem, but gallamine had only marginal effects on the dissociation of [^{3}H]QNB in the forebrain. On the other hand, verapamil exerts only monophasic effects (slowing) on the dissociation of both [^{3}H]NMS and [^{3}H]QNB from heart receptors and gallamine slows the dissociation of [^{3}H]NMS to a similar extent in all three tissues. Thus, it appears that past discrepancies in the literature can be attributed to the tissues and concentrations of gallamine that were used. Furthermore, the biphasic effects of gallamine suggest that there are multiple allosteric regulatory sites associated with muscarinic receptors.

Gallamine is a muscarinic antagonist that discriminates between subpopulations of muscarinic receptors and also regulates binding of muscarinic ligands via an allosteric regulatory site. We have recently shown that these two properties (subpopulation specificity and allosterism) are separate phenomena (1). Indeed, all quaternary muscarinic ligands, including agonists (e.g., carbamylcholine chloride), reversible antagonists (e.g., NMS), and irreversible antagonists (propylbenzilylcholine mustard) seem to share the subpopulation specificity of gallamine to some degree (2, 3). On the other hand, a pharmacologically diverse group of compounds has been found to allosterically modulate muscarinic receptors. These include verapamil (1, 4–6), quinidine (7), and others (8). Most of the studies of allosteric modulation have measured the ability of these agents to slow the rate of dissociation of labeled ligands, usually [^{3}H]NMS, from muscarinic receptors. This strategy is necessary because measurements of association rates or of equilibrium binding are confounded by the competitive interactions of the above-mentioned compounds (1, 6).

There have been relatively few studies of allosteric modulation of the rate of dissociation of [^{3}H]QNB, and the results have been in disagreement. We have reported that, in forebrain membranes, concentrations of gallamine that dramatically slowed the off-rate of [^{3}H]NMS had no effect when the dissociation of [^{3}H]QNB was investigated (9). Another study with forebrain membranes also found no effect of gallamine on the dissociation of [^{3}H]QNB, but it used very low concentrations of gallamine (10). On the other hand, two studies of cardiac muscarinic receptors have reported that 1 mM gallamine does greatly slow the off-rate of [^{3}H]QNB (11, 12).

The present study was undertaken to evaluate whether tissue-related differences exist with regard to the ability of gallamine to allosterically regulate the binding of [^{3}H]QNB to muscarinic receptors. We have found that such differences do exist between tissues. However, a more striking finding is that, within tissues, gallamine exerts biphasic allosteric effects that cannot be explained on the basis of a single allosteric site.

Materials and Methods

Tritiated *l*-QNB (35.2 Ci/mmol) and [^{3}H]NMS (84 Ci/mmol) were purchased from NEN (Dupont; Boston, MA). Unlabeled QNB was from RBI (Natick, MA), whereas gallamine triethiodide, atropine, and verapamil were obtained from Sigma Chemical Company (St. Louis, MO).

Neural membranes were prepared as described previously (13). Briefly, appropriate regions were dissected from the brains of male

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ABBREVIATIONS: NMS, *N*-methylscopolamine; QNB, quinuclidinylbenzilate; PB, sodium-potassium phosphate buffer.

Sprague-Dawley rats (150–200 g) and homogenized in 40 mM PB with 1 mM EDTA (pH 7.4). The supernatant fraction from a low speed centrifugation (3,000 × *g* for 10 min) was subjected to 50,000 × *g* for 20 min and the resulting pellet was resuspended in PB and stored at 70°.

Cardiac membranes were obtained by mincing ventricles from the above-mentioned rats with scissors, then homogenizing the minced ventricles with a biohomogenizer (Biospec Products, Bartlesville, OK) in 40 mM PB at 0° (two times, 30 sec each). The resulting homogenate was filtered through two layers of cheese cloth and centrifuged at 50,000 × *g* for 20 min, and the supernatant was discarded. The pellet was resuspended in PB and stored at −70°.

Binding studies were conducted in 5 mM PB at 22° or 37°, as indicated. Membranes (0.1–0.3 mg of protein in 2 ml) were prelabeled with 1 nM [^{3}H]NMS or [^{3}H]QNB for 30 min. Dissociation of the labeled ligand was initiated by the addition of 1 μM unlabeled QNB, with or without gallamine or verapamil, and the incubation was continued for the indicated times. The assay was terminated by filtration through S&S No. 34 glass fiber filters (Schleicher and Schuell, Keene, NH), followed by two rinses with 40 mM PB (0°). Nonspecific binding was defined by the inclusion of 1 μM atropine. Because gallamine can inhibit nonspecific binding of muscarinic ligands (9, 14), we determined nonspecific binding in the presence of gallamine or verapamil when either concentration exceeded 1 μM. Some experiments were carried out with 0.1 nM labeled ligand, at which concentration the effects of gallamine on nonspecific binding were negligible. Other experiments were carried out with higher concentrations of unlabeled QNB and atropine (10 μM) to initiate dissociation and to determine nonspecific binding. These experiments confirmed that all of the results reported in this paper represent the effects of gallamine and verapamil on *specific* binding.

Results

In our initial studies of the dissociation of [^{3}H]QNB, especially with membranes from the heart and brainstem, we were struck by the fact that low concentrations of gallamine consistently produced results opposite to those obtained at very high (1 mM) concentrations. This phenomenon is illustrated in Fig. 1A. The rate of dissociation of [^{3}H]QNB from cardiac muscarinic receptors is accelerated by 1 μM gallamine and is slowed by 1 mM gallamine. The dissociation of [^{3}H]QNB at 37° is fit reasonably well by a monoexponential function and the half-times of dissociation are 36.5 min without gallamine, 17.1 min with 1 μM gallamine, and 205 min in the presence of 1 mM gallamine. Based on these and similar kinetic studies with [^{3}H]NMS, we chose single time points at which to measure dissociation in the presence of graded concentrations of gallamine. Beginning at about 0.1 μM, gallamine progressively slows the dissociation of [^{3}H]NMS in cardiac membranes (Fig. 1B). Although the potency of gallamine is somewhat lower, very similar effects are produced at brainstem and forebrain receptors (Fig. 2). The interaction between gallamine and QNB is more complex. Gallamine accelerates the dissociation of [^{3}H]QNB from cardiac receptors at low concentrations (beginning at about 0.1 μM), produces no net effect at about 100 μM, and dramatically slows the dissociation at 1 mM (Fig. 1B). A similar interaction occurs between QNB and gallamine in brainstem membranes (Fig. 2A), but the extent of both the accelerating and the slowing of the dissociation is much less in forebrain membranes (Fig. 2B). To investigate the possibility that the different effects of gallamine on [^{3}H]NMS and [^{3}H]QNB were due to the assay temperatures, the interaction between gallamine and [^{3}H]NMS was studied at 37°. Although the rate of dissociation of [^{3}H]NMS was much faster at 37°, the potency and efficacy of gallamine in modulating that dissociation were the same as at 22° (brainstem membranes; data not shown). In cardiac membranes, verapamil produces an allosteric effect that is qualitatively similar to that of gallamine when [^{3}H]NMS is the labeled ligand (Fig. 3). However, unlike gallamine, verapamil failed to accelerate the dissociation of [^{3}H]QNB at any of the concentrations tested (Fig. 3).

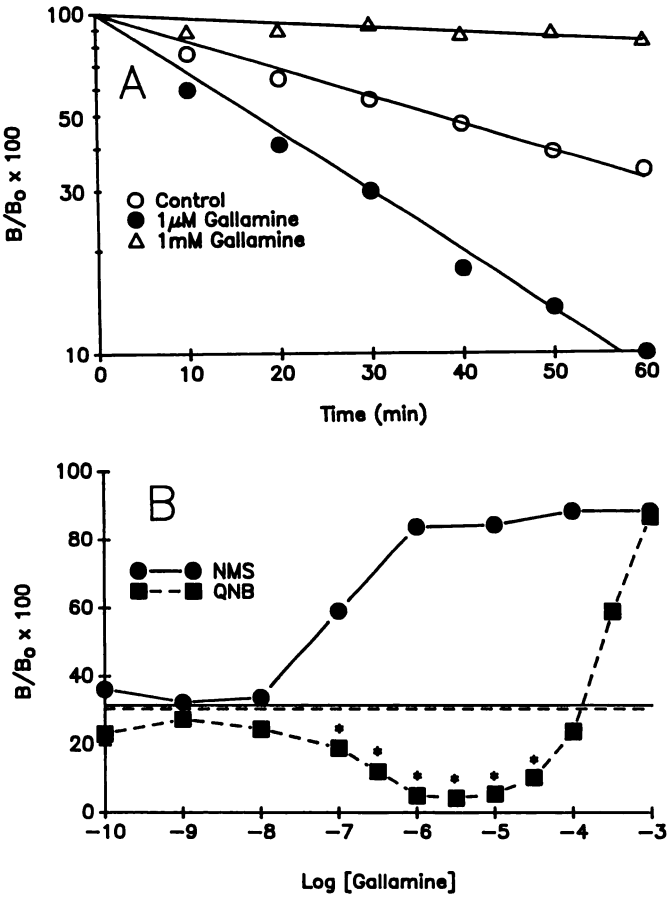

Fig. 1. Allosteric regulation of cardiac muscarinic receptors by gallamine. A, [^{3}H]QNB was equilibrated with cardiac membranes for 30 min at 37°, at which time 1 μM unlabeled QNB was added alone (control) or with the indicated concentration of gallamine. Dissociation was allowed to occur for the indicated times, and the assay was terminated by filtration. The results are expressed as radioactivity specifically bound, as a percentage of the specific binding at time 0. Each *point* is the mean of two experiments, each performed in duplicate. The slopes of the best-fit lines thru the three data sets were found to be significantly different, by means of *F* tests performed on the sums of squares from constrained and unconstrained fits ($p < 0.001$). For half-times of dissociation, see text. B, Experiments were performed and data were expressed as described above. [^{3}H]NMS was allowed to equilibrate with cardiac membranes for 30 min at 22°. Dissociation was initiated by the addition of 1 μM QNB and the indicated concentration of gallamine and the assay was terminated by filtration after 5 min. For [^{3}H]QNB, equilibration was carried out for 30 min at 37° and dissociation proceeded for 60 min. The *solid* and *dashed horizontal lines* represent the specific binding remaining in the absence of gallamine for [^{3}H]NMS (31.6 ± 2.9%) and [^{3}H]QNB (30.4 ± 2.3%), respectively. The *data points* are the mean ± standard error for three experiments, each performed in duplicate. In all cases, the standard errors are smaller than the *symbols*. The *asterisks* indicate concentrations of gallamine that significantly accelerate the dissociation of [^{3}H]QNB ($p < 0.01$, Duncan's multiple range test).

Discussion

These results confirm separate reports from several laboratories (9–12) that have suggested that the allosteric interactions

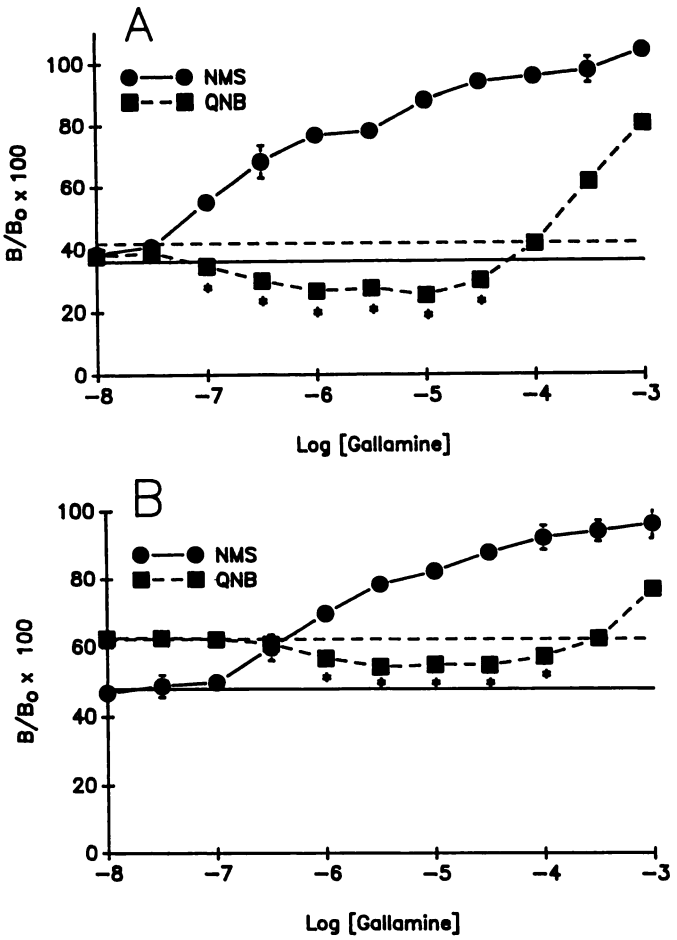

Fig. 2. Allosteric regulation of central nervous system muscarinic receptors by gallamine. Experiments were conducted as in Fig. 1B, except that the time of dissociation for [^{3}H]NMS was 8 min for brainstem membranes (A) and 20 min for forebrain membranes (B). The significance of the *horizontal lines* and the *asterisks* is the same as in Fig. 1B.

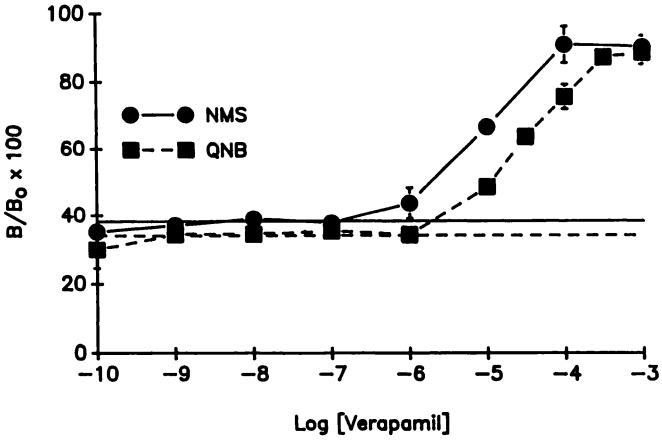

Fig. 3. Allosteric regulation of cardiac muscarinic receptors by verapamil. Experiments were conducted and the data plotted exactly as in Fig. 1B. Verapamil did not accelerate the dissociation of [^{3}H]QNB to a significant extent at any of the concentrations tested.

between gallamine and [^{3}H]QNB may vary between tissues. Thus, 0.3–1.0 mM gallamine dramatically slowed the dissociation of [^{3}H]QNB from cardiac membranes but produced no effect or very modest effects when forebrain membranes were studied (Figs. 1B and 2B). More significant, however, is the finding that at lower concentrations gallamine accelerates the dissociation of [^{3}H]QNB (Figs. 1 and 2). The only other report of allosteric *acceleration* of dissociation of a muscarinic antagonist, to our knowledge, was that of Nedoma *et al.* (11). In that study, high concentrations of ritebronium (500 μM) seemed to accelerate the dissociation of [^{3}H]QNB from rat cardiac membranes. In the same study, gallamine was found to decelerate, but not accelerate, the dissociation of [^{3}H]QNB. It is possible that the differences between that study and the present one stem from the different buffers, tissue sources and preparation, and/or concentrations that were used.

The acceleration and deceleration of the dissociation of [^{3}H]QNB appear to reveal a previously undetected complexity in the allosteric interactions of gallamine. The threshold concentration of gallamine for slowing the dissociation of [^{3}H]NMS is indistinguishable from the threshold for accelerating the dissociation of [^{3}H]QNB (Fig. 1B). It therefore seems likely that the same site is responsible for both effects. Over the range of 1 μM to 1 mM gallamine, the dissociation of [^{3}H]QNB becomes progressively and markedly slower, whereas the rate of dissociation of [^{3}H]NMS is essentially constant (Fig. 1B). Thus, NMS appears to be insensitive to whatever mechanism of action is operating at high concentrations of gallamine. One possibility is that there is a second allosteric site on or associated with muscarinic receptors, occupancy of which (by gallamine) greatly slows the dissociation of QNB. Another possibility is that the effect at high concentrations of gallamine is due to nonspecific membrane perturbations. We consider the second possibility less likely, due to the complete lack of change in the rate of dissociation of NMS over a wide range of gallamine concentrations. The biphasic nature of the effects of gallamine on QNB is not likely to be due to different actions at different subtypes of receptors, because the dissociation plots (i.e., Fig. 1A) are not themselves biphasic and because it is very prominent in the heart.

The tissue-related differences in the potency of gallamine in binding and response assays have led to the suggestion that it is an "M_2-selective" ligand (10, 15). For example, in contrast to pirenzepine, gallamine exhibits cardioselectivity and greater affinity for brainstem receptors than for forebrain receptors (15–17). However, such selectivity undoubtably derives from factors other than the affinity of gallamine for the M_2 binding site. In particular, the subpopulations discerned by quaternary ligands are not differentiated by pirenzepine (3, 18). The allosteric effects of gallamine do appear to differ between M_1 and M_2 sites, although it must be kept in mind that the nature of the labeled ligand used may at times be of greater importance than the receptor subtype that is labeled (14). Such selectivity can be seen as a greater potency in slowing the dissociation of [^{3}H]NMS in M_2 tissues, heart > brainstem > forebrain (Figs. 1 and 2; also see Ref. 19). The same order is apparent for both potency and efficacy when dissociation of [^{3}H]QNB is examined. It has been reported that guanine nucleotides can reduce the ability of gallamine to modulate the dissociation of [^{3}H]NMS in brainstem, but not cortical, membranes (20), again suggesting differences in the allosteric interactions of gallamine with M_1 and M_2 receptors. However, we have so far detected no effect of guanine nucleotides on the modulation by gallamine of dissociation of [^{3}H]QNB in cardiac membranes. Another cardioselective muscarinic antagonist, methoctramine, has recently been reported to interact both competitively and allos-

terically with cardiac muscarinic receptors (21). On the other hand, AF-DX 116 seems to be a purely competitive cardioselective antagonist (17).

Verapamil did not exert biphasic effects on the dissociation of [^{3}H]QNB (Fig. 3), possibly due to not interacting with one of the allosteric sites. Alternatively, verapamil may interact with both sites, but the conformational changes induced in the receptor may be very different from those induced by gallamine. It is difficult to choose between these and a number of other speculative models, because it has not yet become possible to determine whether any two muscarinic allosteric modulators compete with each other at the same allosteric site (8). We (1) and others (6) have noted previously that there are significant difficulties associated with accurately quantitating the allosteric interactions at muscarinic receptors, due to concomitant competitive interactions at the classical muscarinic binding site. Although equations for a purely allosteric model have been thoroughly described (19, 22) and it is not difficult to rule out the strictly competitive model, the model for a mixed (competitive plus allosteric) interaction is much more complicated. For example, a mixed competitor could allosterically modify its own ability to bind to the classical site. Until agents that interact only at the allosteric site are discovered, it seems that the best way to examine muscarinic allosteric events in isolation from the competitive components is to measure rates of dissociation of labeled ligands in the presence and absence of allosteric modulators.

The value of allosteric modulation of receptor function has been well illustrated by the γ-aminobutyric acid-benzodiazepine receptor complex (23). That is, allosteric modulators may tune receptor sensitivity up or down, in terms of the potency or efficacy of the endogenous agonist; the nature of allosteric action does not preclude temporal patterning, as do directly acting agonists and antagonists; and allosteric modulation has inherent safety features (23). The complex allosteric effects observed in this study may have a variety of physiological correlates, whereas the qualitative differences between the actions of two allosteric modulators (gallamine and verapamil) suggest a potential for pharmacological selectivity. Thus, in spite of the present difficulty in accounting for the competitive component of gallamine's antagonism, it is not unlikely that allosteric modulation of muscarinic function may eventually offer therapeutic benefits (24).

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Send reprint requests to: John Ellis, Neuroscience Research Unit, Department of Psychiatry, University of Vermont College of Medicine, Burlington, VT 05405.