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COMPETITIVE INTERACTION OF GALLAMINE WITH MULTIPLE MUSCARINIC RECEPTORS

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Abstract—Gallamine, a cholinergic antagonist at the (nicotinic) neuromuscular junction, possesses antimuscarinic potency in several systems. We report here that gallamine inhibited the binding of [^{3}H]quinuclidinyl benzilate (QNB) in a competitive manner in the brainstem and forebrain of the rat. The occupancy curves derived from these studies suggest that gallamine has widely varying affinities for different subpopulations of muscarinic receptors, a finding which sets gallamine apart from classical muscarinic antagonists such as atropine and QNB. The greatest difference in affinities for gallamine occurred in the brainstem, where the data could be satisfactorily fitted to a two-site model, with 77% of the receptors having high affinity (K_d = 25 nM) and 23% low affinity (93 μM). Further, these affinities displayed rank order correlation with those of carbachol (an agonist), although gallamine has not, so far, displayed agonist (or partial agonist) activity. The finding that antagonists as well as agonists can display multiple affinities for muscarinic receptors suggests that there are fundamental differences among subpopulations of these receptors.

It is now well established that a particular neurotransmitter may interact with more than one receptor. Experiments concerning adrenergic, cholinergic, dopaminergic, and opiate systems yield results which are best explained by the postulation of discrete subtypes of receptors. Just as the adrenergic classification has been subdivided beyond and α and β designations, the terms nicotinic and muscarinic no longer completely specify the types of cholinergic receptors. There is general agreement that at least three types of muscarinic cholinergic receptors exist in the brain of the rat [1, 2]. Classical muscarinic antagonists such as quinuclidinyl benzilate (QNB) and atropine do not distinguish the difference between these subpopulations, whereas agonists such as oxotremorine, acetylcholine, and carbachol do. This contrasts with the dopaminergic system, for example, where both agonists and antagonists detect heterogeneity in the receptor population [3]. This qualitative difference between the interactions of agonists and antagonists at muscarinic receptors suggested that the differences between the muscarinic subpopulations might lie in effector coupling rather than in structural differences of the receptive components [4]. Antagonists, which do not trigger the effector, would be unaffected by these coupling variations.

In this paper we report that gallamine, a nicotinic antagonist with antimuscarinic potency in several systems, interacts competitively with the tritiated ligand QNB at the muscarinic receptor. Furthermore, gallamine discriminates subpopulations of muscarinic acetylcholine receptors, although it shows no agonist or partial agonist character. These findings suggest that there may be differences among the receptive components of the muscarinic subpopulations.

MATERIALS AND METHODS

Materials. Tritiated 3-quinuclidinyl benzilate was purchased from the Amersham Corp. (Arlington Heights, IL) as the L-isomer (L-QNB, 44 Ci/mmole). Carbachol was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Gallamine triethiodide was purchased from K & K Laboratories (Plainview, NY).

Tissue preparation. Neural membranes were prepared as described previously [2]. Briefly, appropriate regions were dissected from the brains of male Sprague–Dawley rats (150 g) and homogenized in 40 mM Na–K phosphate buffer (PB) with 1 mM EDTA. The supernatant fraction from a low-speed centrifugation (3,000 × *g*, 10 min) was subjected to 50,000 *g* for 20 min, and the resulting pellet was resuspended in PB, flash frozen in liquid N_2, and stored at −70°. No deterioration of binding parameters was noticeable after 1 month of such storage.

Binding assays. The binding of gallamine and carbachol to the muscarinic receptor was inferred from the displacement of tritiated QNB. The protocol for these assays has been described [2]. The receptor concentration was maintained at 5 pM, which is sufficiently less than the K_d for L-QNB (50 pM) in this system [2]. Incubations were conducted for 90 min at room temperature, conditions under which equilibrium values are attained, and were terminated by filtration through glass fiber filters.

Protection experiments. In some experiments, a preincubation was carried out to block a portion of

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the receptors with unlabeled QNB. For these studies, receptors were incubated at a concentration of 400 pM for 30 min at room temperature under one of the following conditions: (a) 0.5 nM unlabeled D,L-QNB alone to block receptors randomly; (b) 2 nM unlabeled D,L-QNB plus either 0.1 mM (forebrain) or 5 μM (brain stem) carbachol, to selectively protect high-affinity receptors; (c) carbachol alone at the same concentrations as in (b), to control for desensitization-like phenomena; or (d) no ligand present (control). The incubation was terminated by centrifugation at 45,000 *g* for 10 min, and the pellet was washed twice. The washing procedure consisted of mild homogenization in fresh buffer, incubation for 15 min at room temperature to ensure dissociation of carbachol (but not QNB, which dissociates very slowly) from the receptor, and centrifugation. This treatment results in a preparation with greater affinity toward carbachol by virtue of the preferential elimination of sites with low agonist affinity, while the affinity toward QNB is unchanged.

Data analysis. Binding data were analyzed according to a model in which unlabeled competitors distinguish two populations of receptors, while QNB binds homogeneously, in agreement with the observed characteristics of muscarinic receptors [2]. The parameters for the best fit of this two-site model to the data were obtained by the use of the nonlinear curve-fitting routine MLAB, available from NIH, on the DEC-10 system at the University of Rochester.

RESULTS

Binding of typical agonists and antagonists. The affinities of some typical muscarinic agonists (carbachol, oxotremorine, arecoline) and antagonists (QNB, atropine) for the brainstem and the forebrain are listed in Table 1. It is readily apparent that the three agonists possessed greater relative affinity for the brainstem, while QNB and atropine showed little preference for either region. Gallamine showed significant differences in affinities between these regions despite the fact that studies to date have described only antagonist properties in the muscarinic system [5–8]. Although gallamine is an antagonist, it possesses the regional selectivity characteristic of agonists.

Table 1. Affinities of agonists and antagonists for the brainstem and the forebrain*

	IC_{50} (nM)	
	Brainstem	Forebrain
Carbachol	790	6900
Oxotremorine	88	311
Arecoline	600	5000
Gallamine	84	510
Atropine	2.3	1.2
QNB	0.19	0.30

* Values were determined by the inhibition of the binding of 50 pM [^{3}H]-L-QNB.

In our hands gallamine blocks the muscarinic stimulation of the turnover of phosphatidyl inositol in brain slices, but has no stimulatory effect of its own [9].

Binding of gallamine to muscarinic receptor. The double-reciprocal plot in Fig. 1 reveals that the inhibition by gallamine of the binding of QNB was competitive and multiphasic. The solid line through the circles was the best fit to a two-site model for which the parameters are given in the legend to Fig. 1. It should be emphasized that this plot does not give an accurate estimate of K_L under these conditions and was chosen for the purpose of illustrating the competitive nature of the interaction. The other parameters (α, K_H) are in good agreement with the values found in Table 2.

Table 2. Parameters of the binding of gallamine and carbachol to neural membranes, with and without selective elimination of receptors with low affinity for carbachol*

	IC_{50} (μM)	n_H	α	K_H (μM)	K_L (μM)
Brainstem					
Gallamine					
Untreated	0.084		0.77 ± 0.01	0.025 ± 0.002	93 ± 38
Treated	0.044		0.95 ± 0.01	0.022 ± 0.001	98 ± 71
Carbachol					
Untreated	0.79	0.50			
Treated	0.15	0.49			
Forebrain					
Gallamine					
Untreated	0.51		0.76 ± 0.01	0.15 ± 0.01	46 ± 10
Treated	0.14		0.51 ± 0.09	0.014 ± 0.009	0.44 ± 0.12
Carbachol					
Untreated	6.9	0.48			
Treated	0.96	0.45			

* Abbreviations: n_H, Hill coefficient; K_H, dissociation constant for high-affinity site; K_L dissociation constant for low-affinity site; and α, fraction of sites with high affinity. The parameters α, K_H, and K_L (± S. E.) were determined by analysis of the inhibition of QNB binding by the MLAB (nonlinear curve-fitting) system, available from NIH, on the DEC-10 computer at the University of Rochester. The treatment of the receptors is described in Materials and Methods.

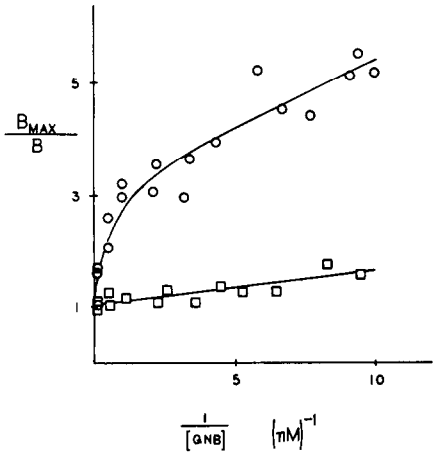

Fig. 1. Double-reciprocal plot of the specific binding of [^{3}H]-L-QNB to forebrain membranes in the presence (○) and absence (□) of 30 μM gallamine. The intercept of 1.0 on the B_{max}/B axis is indicative of a competitive interaction. The line through the circles (+ gallamine) represents the best fit to a two-site model using the non-linear curve-fitting routine MLAB. The parameters for the best fit were: $\alpha = 0.72 \pm 0.11$, $K_H = 0.303 \pm 0.133$ μM, and $K_L = 36.2 \pm 934$ μM. Except for K_L, which is not accurately determinable from this kind of plot, these values are in agreement with the (untreated) data in Table 2.

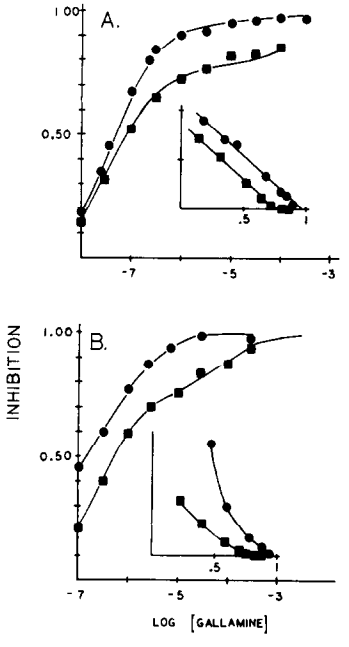

Fig. 2. Plots of inhibition by gallamine of the binding of 50 pM [^{3}H]-L-QNB in membranes from the brainstem (A) and the forebrain (B), with (●) and without (■) the selective elimination of sites with low affinity toward carbachol (see Materials and Methods). The best-fit data are presented in Table 2. The insets show Scatchard-analog replots of the data (abscissa, inhibition; ordinate, inhibition per concentration).

Effect of enrichment of high-affinity carbachol sites on gallamine binding. The ability of gallamine to compete for the binding of QNB in the brainstem and forebrain is presented graphically in Fig. 2 (A and B). In each region the binding studies were conducted on untreated membranes as well as on membranes that had been treated to selectively eliminate receptors with low affinity for carbachol (see Materials and Methods). It is apparent that gallamine had greater affinity for the treated membranes and that the brainstem receptors were rendered homogeneous with respect to affinity for gallamine by the treatment. In contrast, the same treatment did not eliminate the heterogeneity seen by carbachol in the brainstem, in agreement with the suggestion that there are more than two sites for carbachol [1, 2]. The affinity of the forebrain for gallamine was also increased (Fig. 2) by the above treatment, although heterogeneity remained in the occupancy curves of both gallamine and carbachol.

DISCUSSION

The main finding in the present work is that gallamine interacts competitively at central muscarinic receptors and, despite a lack of agonist activity, distinguishes subpopulations of muscarinic receptors. The order of affinity of gallamine and carbachol for subpopulations of muscarinic receptors is similar. Both drugs possess greater affinity for the brainstem than for the forebrain, and the affinity of each is enhanced by the elimination of receptors with low affinity for carbachol (see Table 2). Gallamine and carbachol do not, however, subdivide the receptors in an identical manner since the treatment which selectively eliminates receptors with low affinity for carbachol renders the brainstem homogeneous with respect to gallamine but not with respect to carbachol (see Table 2).

The forebrain retains heterogeneity toward both gallamine and carbachol subsequent to the treatment which eliminates receptors with low affinity for carbachol. It appears that the forebrain has a site of intermediate affinity for gallamine (approximately 0.4 μM—see Table 2, "treated") which is not present in the brainstem. Therefore, the proportion of high affinity sites seen by a two-site model can decrease along with the IC_{50} (Table 2), reflecting the proportions of the remaining sites. Although the two-site fit is therefore inaccurate, it provides a good estimate of the spread of the affinities, especially when coupled with a pharmacological treatment such as the enrichment of high-affinity sites. The existence of more kinds of gallamine sites in the forebrain than in the brainstem is reminiscent of the studies of Hammer *et al.* [10], who found the muscarinic antagonist, pirenzipine, to bind heterogeneously in the forebrain and homogeneously in the brainstem.

Clark and Mitchelson [8] have suggested that gallamine interacts noncompetitively at muscarinic receptors in the heart. Our demonstration of correlation in the order of affinities between gallamine sites and carbachol sites (Table 2) bolsters the conclusion from Fig. 1 of a competitive interaction in the brain regions examined. The postulation that the negative inotropic effect of carbachol is coupled to

both high- and low-affinity muscarinic receptors in the heart, together with gallamine binding similar to our description, predicts curved Arunlakshana–Shild plots as observed by Clark and Mitchelson. It is possible, however, that brain and heart simply differ with regard to the action of gallamine.

The relationship between the regional variations in affinity of a given muscarinic ligand and the heterogeneity of the binding of that ligand within a single region appears to be complex. Birdsall *et al.* [1] have stated that the postulation of three subtypes of receptors adequately explains the heterogeneity both within and among regions. However, the data in Table 2 suggest a complex relationship between the subtypes that are defined by gallamine and carbachol in the brainstem and forebrain. From one point of view there seem to be two sites for gallamine in the brainstem, but at least three in the forebrain (see Table 2 and Results). Yet there also seem to be more than two sites for carbachol in the brainstem (Table 2) and, therefore, gallamine must bind to two of the sites defined by carbachol with equal affinity. If there were only a total of three sites in the rat brain, and gallamine possessed identical affinity for two of them, then there could only be two sites for gallamine in the forebrain also. A less complicated case can be stated for the binding of pirenzipine, which is homogeneous in the medulla-pons, yet heterogeneous in the forebrain [10]. If the receptor subtypes are the same in the two regions, pirenzipine should discriminate between them in both areas. This argument is especially strong since the medulla-pons possesses a very even distribution of subtypes (30%–42%–28% [1]). There is very strong empirical evidence that muscarinic ligands which exhibit regional variations in affinity are the same ligands which bind heterogeneously within a given region. Nonetheless, it seems that even the postulation of three sites is not sufficient to explain the accumulated data.

Several studies have compared central versus peripheral potencies of muscarinic antagonists as a measure of intrinsic differences between types of muscarinic receptors [11–14]. Those exhibiting the greatest selectivity (or heterogeneity, in binding studies) have affinities considerably lower than atropine, as suggested by Fisher *et al.* [11]. The relatively low affinity of gallamine is in agreement with this trend.

Classification of muscarinic subpopulations has been hampered by the lack of ligands with a strict pharmacological specificity. While it has been recognized that agonists discriminate receptor subtypes, the order of potency of all agonists tested to date is the same [1]. As mentioned above, this phenomenon lends support to the coupling theory of receptor heterogeneity [4, 15]. However, the very fact of effector coupling makes agonists poor choices in the pharmacological classification of receptors. The affinities of antagonists are better criteria by which to define receptors [16]. The existence of antagonists which distinguish muscarinic subpopulations implies that there are differences other than (or, more likely, in addition to) coupling between the subpopulations. These differences may be minor and, indeed, may be due to receptor conformation or local membrane environment rather than to differences in the primary structure of the receptors. Nevertheless, such differences signify the potential for pharmacological dissection of the multitude of physiological [17] and behavioral [18] phenomena in which muscarinic cholinergic systems are implicated.

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