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Heterogeneity of Binding Sites on Cardiac Muscarinic Receptors Induced by the Neuromuscular Blocking Agents Gallamine and Pancuronium

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SUMMARY

The binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to cardiac muscarinic receptors was inhibited not only by classical muscarinic antagonists but also by nicotinic blocking agents and inhibitors of acetylcholinesterase. Gallamine, pancuronium, ambenonium, and decamethonium were the most potent of these agents examined. All of the nicotinic antagonists with significant muscarinic receptor activity had two or three quaternary nitrogens, and the potency of a series of these compounds was a function of the distance between quaternary nitrogens. The effects of gallamine and pancuronium were studied in detail because these neuromuscular blocking agents showed heterogeneity in their binding to cardiac muscarinic receptors, whereas classical muscarinic antagonists such as QNB and atropine did not. Gallamine did not compete for all of the [^{3}H]QNB binding sites on atrial membranes, but left at least 20% of [^{3}H]QNB binding unaffected. Curves of pancuronium competition for [^{3}H]QNB binding were shallow, consistent with two binding sites for pancuronium, with approximately 20% having low affinity. Additionally, in the presence of gallamine or pancuronium, [^{3}H]QNB binding sites were no longer homogeneous, and Scatchard plots became nonlinear. Guanine nucleotides did not alter the effect of gallamine or pancuronium on [^{3}H]QNB binding. Gallamine and pancuronium showed no agonist activity but, like atropine, completely antagonized muscarinic receptor-mediated inhibition of cyclic AMP formation. However, differences in the behavior of gallamine and atropine suggested that gallamine was not a purely competitive antagonist. Gallamine did not protect against receptor alkylation by propylbenzilylcholine mustard, and [^{3}H]QNB dissociation was apparently slowed by gallamine. We interpret our data to suggest that gallamine not only competes for [^{3}H]QNB binding sites, but also binds at a secondary site on the receptor, forming a ternary complex with [^{3}H]QNB. Heterogeneity in ligand binding is proposed to result from the dual actions of gallamine and pancuronium as ligands at both primary and secondary sites on the cardiac muscarinic receptor.

INTRODUCTION

Muscarinic receptors mediate a variety of responses regulated through the parasympathetic nervous system. Physiological and pharmacological data suggest that there are differences in the cardiac muscarinic receptors regulating rate, contractility, and conduction (1–4), as well as differences in the muscarinic receptors in heart and ileum (4–6). Despite these findings there has been no formal subclassification of muscarinic receptors. If muscarinic receptor subtypes exist, differences in receptors should be demonstrable in receptor binding studies using radiolabeled antagonists. However, most studies of [^{3}H]QNB[2] or [^{3}H]N-methylscopolamine binding have shown that antagonists interact with only a homogeneous population of cardiac muscarinic receptors under physiological conditions (7–10).

There are several neuromuscular blocking agents that produce tachycardia when used clinically, apparently through vagal blockade. One of these agents is gallamine (Flaxedil), which has been extensively analyzed in physiological studies. It has been shown that gallamine is a more potent antagonist of the effects of ACh in heart than in ileum or at other peripheral parasympathetic sites (11–13), that gallamine produces selective blockade of electrophysiological changes mediated through a sub-

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[2] The abbreviations used are: QNB, (−)-quinuclidinyl benzilate; ACh, acetylcholine; Gpp(NH)p, 5′-guanylylimidodiphosphate; BTM, bis-trimethylammonium; PBCM, propylbenilylcholine mustard.

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set of muscarinic receptors in the ganglion (14), and that gallamine prevents the effects of ACh on atrioventricular conduction at much lower concentrations than those needed to block sinus node automaticity (3). A similar pattern of selectivity has been shown for pancuronium (14, 15). In this study we sought to determine whether gallamine and related neuromuscular blocking agents interact directly with cardiac muscarinic receptors and whether these agents might select for and demonstrate pharmacologically different populations of muscarinic receptors. Preliminary reports of some of these data have appeared previously (16, 17).

MATERIALS AND METHODS

Atropine sulfate, methylatropine nitrate, *d*-tubocurarine chloride, hexamethonium bromide, decamethonium bromide, physostigmine sulfate, neostigmine methylsulfate, and Gpp(NH)p were from Sigma Chemical Company (St. Louis, Mo.). Echothiophate iodide (Phospholine) was from Ayerst Labortories (New York, N. Y.). Ambenonium chloride (Mytelase, WIN 8077) was a gift from Sterling-Winthrop (New York, N. Y.). Gallamine triethiodide was from ICN (Plainview, N. Y.). Pancuronium bromide and ORG. NC 45 were from Organon Pharmaceuticals (West Orange, N. J.). The polymethylene BTM compounds and PBCM were provided by Dr. Palmer Taylor, University of California, San Diego. *d*- and *l*-Benzetimide were gifts from Dr. Steven Mayer, niversity of California, San Diego. [^{3}H]QNB (33.1 Ci/mmole) was from New England Nuclear Corporation (Boston, Mass.).

Male Sprague-Dawley rats (150–300 g) were killed by cervical dislocation. Atria were rapidly removed and placed in 30 volumes of an ice-cold Krebs salt solution (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, and 10 mM glucose) buffered with 25 mM sodium 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (pH 7.4). The tissue was homogenized with three 10-sec bursts (at 30-sec intervals) of a tissue homogenizer (Tissumizer, Tekmar Corporation) at maximal speed. The homogenates were centrifuged at 20,000 × *g* for 10 min, and the pellet was resuspended in the same buffer to give a final assay concentration of 0.6–0.8 mg of original tissue (wet weight) (30–40 μg of protein) per milliliter. For competition experiments, this membrane preparation was incubated with 0.15–0.2 nM [^{3}H]QNB in a volume of 2–4 ml at 35°. At the end of a 40-min incubation, 10 ml of a hypotonic Krebs buffer was added to each tube, and the contents were filtered through glass-fiber filters (Whatman GF/C). The tubes and filters were then washed with an additional 20 ml of buffer, and the radioactivity retained on the filter was determined by liquid scintillation counting. Nonspecific binding was defined as that occurring in the presence of 1 μM atropine and was less than 10% of the total binding.

Data were analyzed using a weighted nonlinear least-squares computer curve-fitting program (18) with the VAX II computer at the University of California, San Diego, and analyzed according to a model for the mass-action binding of the radioligand to one or more binding sites. Statistical differences between models were determined by comparing the residual variances of fits to the data. A model for two binding sites was accepted only when it fit the data significantly ($p < 0.05$) better than a model for a single binding site. Saturation binding curves were similarly analyzed to obtain the affinity constant (K_D) and maximal number of binding sites (B_{max}) for [^{3}H]QNB.

The association rate constant (k_1) was calculated from the initial rate of binding (K_{obs}) according to the equation

$$k_1 = \frac{(K_{obs} - k_{-1})}{[^3\text{H}]\text{QNB}}$$

The dissociation rate constant (k_{-1}) was determined following the addition of excess atropine, QNB, or gallamine at the end of a 40-min equilibration.

Protein concentrations were determined by the method of Bradford (19). Cyclic AMP determinations were carried out in murine atria, incubated and assayed as described previously (20).

RESULTS

Characteristics of [^{3}H]QNB binding to rat atrial membranes. The basic parameters of [^{3}H]QNB binding were consistent with those described by others (7–9, 21) and are thus described only briefly here. Binding was linear with protein concentration in the range of 20–200 μg/ml. Specific binding was saturable, whereas the nonspecific binding component increased linearly with [^{3}H]QNB concentration. The averaged values obtained from Scatchard analyses of [^{3}H]QNB binding isotherms were $K_D = 57 \pm 6$ pM and $B_{max} = 779 \pm 110$ fmoles/mg of protein. Scatchard plots of [^{3}H]QNB binding were linear, with average Hill coefficients of 0.98 ± 0.02. Scatchard plots with up to 20 QNB concentrations were best fit by a one-site model, suggesting that [^{3}H]QNB binds to a homogeneous population of muscarinic receptors in heart.

The specific binding of 0.15 nM [^{3}H]QNB to rat atrial membranes at 35° was maximal at 40 min and was maintained at this level for at least an additional 60 min. The association rate constant (k_1) calculated from K_{obs} was 3.8×10^8 M^{-1} min^{-1}. The dissociation of [^{3}H]QNB was first-order, with a half-life of 38–44 min ($k_{-1} = 1.6$–1.8×10^{-2} min^{-1}). The kinetically determined dissociation constant (k_{-1}/k_1), calculated from the average of these values, was 45 pM, in close agreement with the K_D value obtained from equilibrium binding experiments.

Antagonism by muscarinic and nicotinic blocking agents and by cholinesterase inhibitors. The relative potencies of muscarinic antagonists in competing with [^{3}H]QNB were comparable to those reported by others (Fig. 1; Table 1).The Hill coefficients obtained for unlabeled QNB, atropine, methylatropine, and dexetimide were close to unity, as would be expected for a homogeneous and noninteracting population of [^{3}H]QNB binding sites. The *l*-isomer of benzetimide (levetimide) had approximately one-thousandth the potency of the *d*-isomer (dexetimide), demonstrating stereospecificity of the [^{3}H]QNB binding sites in atria.

Several nicotinic blocking agents were also examined in competition experiments (Fig. 1; Table 1). The most potent was pancuronium, with an IC_{50} of 2.6 μM. Other nicotinic agents with significant muscarinic receptor affinity were gallamine, ORG. NC 45, and decamethonium. Ambenonium, a cholinesterase inhibitor with vagolytic and neuromuscular blocking activity (23), was also a potent competitor for [^{3}H]QNB binding sites in atrium, as we have recently reported (24). Muscarinic receptor affinity was greater for agents directed at neuromuscular rather than ganglionic nicotinic sites. This was demonstrated by the increase in potency seen in a series of BTM compounds, which differed only in the number of methylene groups separating the quaternary nitrogens (Fig. 2). Decamethonium, which has selectivity for neuromuscular sites, and hexamethonium, which acts primarily at ganglionic sites, are included in this series.

The pancuronium competition curve, as shown in Fig. 1, was broad, with a Hill slope (n_H) of 0.61. The data were best fit by a two-site model assuming a single K_D

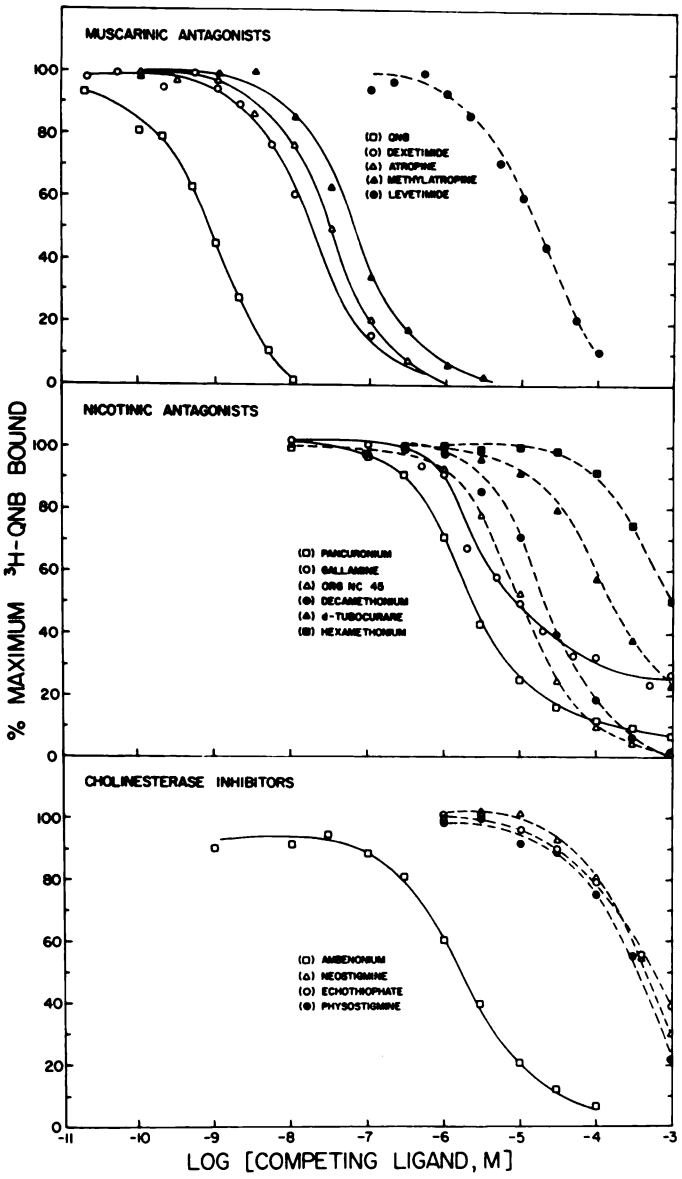

FIG. 1. *Competition of muscarinic and nicotinic antagonists and cholinesterase inhibitors for [³H]QNB binding sites*

All competition assays were carried out under similar conditions, using a concentration of [³H]QNB 0.15–0.2 nM.

value for QNB. The binding parameters calculated for the data shown in Fig. 1 were 76% of sites with a K_i for pancuronium of ~2 μM and 24% with a K_i of ~15 μM. Similar data were obtained in other experiments. The stable guanine nucleotide Gpp(NH)p (100 μM) did not steepen this curve or alter the K_i values for pancuronium. This contrasts with agonist competition curves, which are shifted rightward and steepened in the presence of guanine nucleotides[3] (21).

The effect of gallamine was remarkable in that approximately 20% of specific [³H]QNB binding was retained at maximally effective gallamine concentrations (Fig. 1). This residual binding was not due to increases in nonspecific binding, since nonspecific binding was not

[3] J. H. Brown and J. Dunlap, manuscript in preparation.

TABLE 1

Relative potencies of drugs in competing for [³H]QNB binding to rat atrial membranes

The values given here were calculated from the curves shown in Fig. 3, which are representative of data obtained in two to five experiments. The apparent dissociation constants ($K_{D_{app}}$) for antagonists with n_H ~1 can be calculated from the IC_{50} values by the method of Cheng and Prusoff (22); at the [³H]QNB concentration used (~0.2 nM), the IC_{50} value listed above would be 4–5 times the $K_{D_{app}}$. Hill slopes were not calculated for antagonists of very low affinity, since only partial competition curves were available.

Drug	IC_{50}	n_H
Muscarinic antagonists		
QNB	0.85 nM	0.95
Dexetimide	16 nM	0.94
Atropine	28 nM	0.94
Methylatropine	57 nM	0.99
Levetimide	14 μM	0.99
Nicotinic antagonists		
Pancuronium	2.6 μM	0.61
Gallamine	5.4 μM	0.79
ORG. NC 45	11 μM	1.01
Decamethonium	22 μM	0.98
d-Tubocurarine	140 μM	—
Hexamethonium	990 μM	—
Cholinesterase inhibitors		
Ambenonium	2 μM	0.73
Physostigmine	350 μM	—
Neostigmine	410 μM	—
Echothiophate	600 μM	—

elevated by gallamine at concentrations up to 1 mM. In additional experiments we examined gallamine competition curves at different [³H]QNB concentrations (Fig. 3). As the concentration of [³H]QNB was increased, the gallamine competition curve showed an expected shift rightward. The K_i values for gallamine were calculated

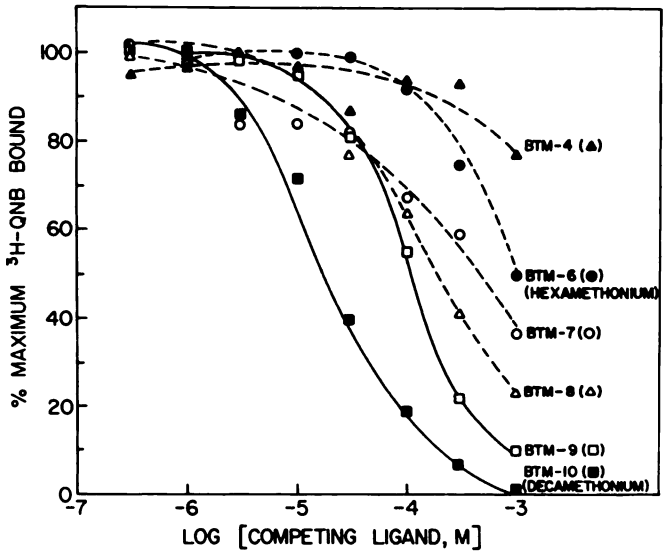

FIG. 2. *Competition of BTM ammonium compounds for [³H]QNB binding*

A series of BTM compounds differing only in the number (n) of methylene groups between the quaternary nitrogens (BTM-n) were tested. Decamethonium (BTM-10) and hexamethonium (BTM-6) are shown again here for comparative purposes.

from the IC_{50} value obtained from each of these curves; all of these values were ~2 μM, a finding consistent with a competitive interaction between gallamine and QNB. Unexpectedly, however, there was an increase in the amount of residual or gallamine-insensitive binding as the [^{3}H]QNB concentration was raised.

Characterization of antagonism by gallamine and pancuronium. Classical muscarinic antagonists such as atropine are competitive inhibitors which decrease the apparent affinity of muscarinic receptors without lowering receptor number. The same should be true of the neuromuscular blocking agents if they simply exclude radioligand from binding to receptor sites on atrial membranes. Saturation binding isotherms were carried out in the presence of various concentrations of atropine, gallamine, and pancuronium. Figure 4 shows Scatchard plots of the [^{3}H]QNB binding isotherms obtained in the presence of increasing concentrations of gallamine. With 3 μM gallamine there was an apparently competitive interaction between [^{3}H]QNB and gallamine, i.e., a 3-fold increase in the K_D with no decrease in B_{max} for

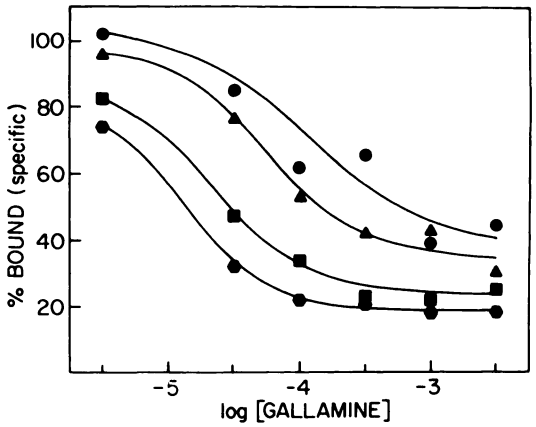

FIG. 3. *Gallamine competition at different [^{3}H]QNB concentrations*

The [^{3}H]QNB concentrations were 0.2 nM (●), 0.4 nM (■), 1.2 nM (▲), and 1.9 nM (●). The 100% value (not shown for the lower [^{3}H]QNB concentrations) was defined as the amount of specific [^{3}H]QNB binding in the absence of gallamine.

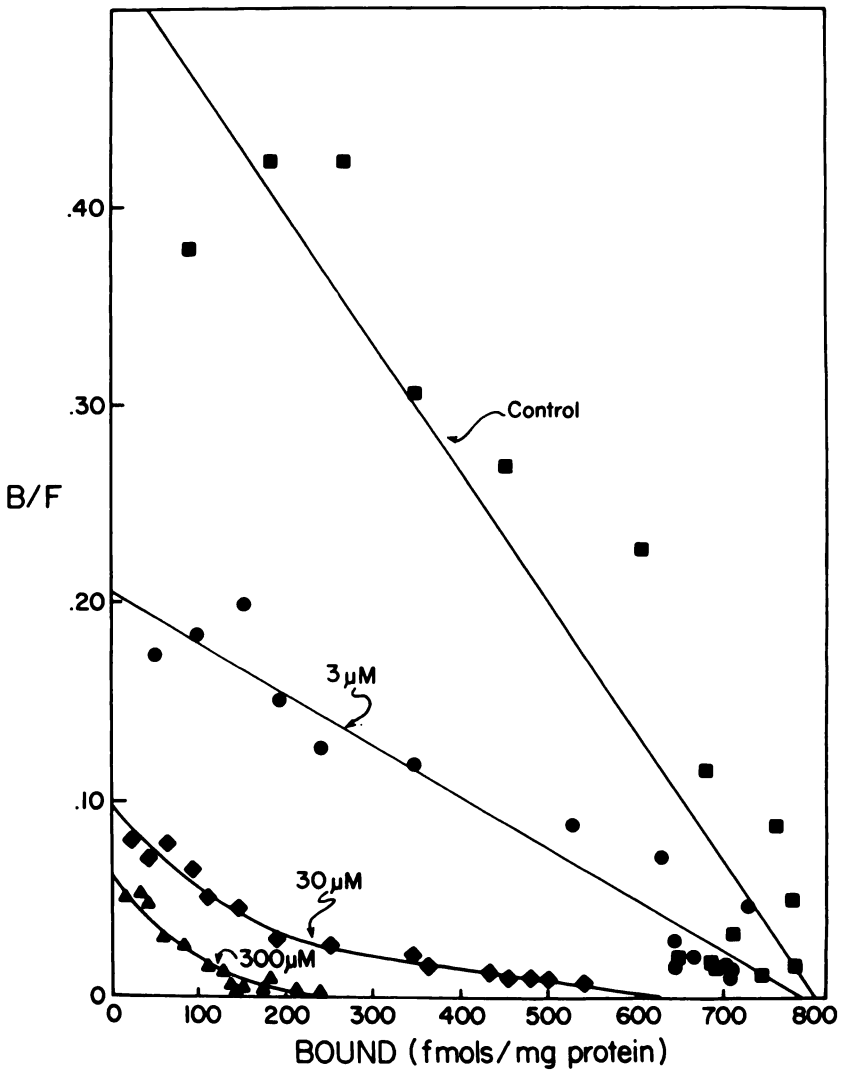

FIG. 4. *Scatchard plots of [^{3}H]QNB binding in the presence of increasing concentrations of gallamine*

[^{3}H]QNB binding isotherms were fitted to one- or two-site models. A one-site model gave the best fit for the control and 3 μM gallamine data; a two-site model gave a better fit to the data obtained in the presence of 30 and 300 μM gallamine. The curves shown on the Scatchard plots are hand-drawn. The parameter estimates for [^{3}H]QNB binding in the presence of 30 μM gallamine were K_1 = 0.055 nM, R_1 = 19.6%; K_2 = 1.07 nM, R_2 = 80.4%.

[^{3}H]QNB. However as the gallamine concentration was increased above its IC_{50}, Scatchard plots became curvilinear. The [^{3}H]QNB binding isotherms seen in the prssence of 30 μM gallamine were analyzed in three separate experiments; in each experiment the one-site model, which fit [^{3}H]QNB binding data obtained under control conditions or with 3 μM gallamine, was now inadequate to describe [^{3}H]QNB binding. Significant improvement in fit was achieved using a two-site model. The parameter estimates from the three experiments showed that between 80% and 88% of the [^{3}H]QNB binding sites were of 10- to 20-fold lower apparent affinity in the presence of 30 μM gallamine. The remaining 12–20% of [^{3}H]QNB binding was of unchanged (control) or slightly higher affinity. The guanine nucleotide Gpp(NH)p (100 μM) had no effect on the binding curves obtained in the presence of 30 μM gallamine. Pancuronium (30 μM) also produced nonlinear Scatchard plots of [^{3}H]QNB binding (Fig. 5) which were best fit by a model in which 20% of the binding sites had a K_D close to normal, whereas 80% had a markedly increased K_D for [^{3}H]QNB.

The effects of gallamine and pancuronium were contrasted with that of atropine which, even at high concentrations, decreased the apparent K_D of all [^{3}H]QNB binding sites in atria and did not produce heterogeneity in [^{3}H]QNB binding (data not shown). There were several other differences in the actions of gallamine and atropine on atrial muscarinic receptors. As mentioned earlier, [^{3}H]QNB dissociated from the receptor with a half-time of approximately 40 min. In these experiments we determined the off-rate by adding a sufficient excess of unlabeled QNB or of atropine to prevent the dissociated [^{3}H]QNB from rebinding. When a comparably high concentration of gallamine (100 times the IC_{50}) was used in place of atropine or QNB, the dissociation rate was 4.6×10^{-3} min^{-1} ($t_{1/2}$ = 152 min), far slower than that obtained with atropine. The curves of [^{3}H]QNB dissociation following addition of atropine and gallamine are compared in Fig. 6.

The mechanism of action of gallamine was also distinguished from that of the competitive antagonist atropine in a "protection" experiment using PBCM, a muscarinic receptor antagonist which irreversibly binds to the receptor through an alkylation reaction (25). Atrial membranes were incubated for 60 min with the mustard alone or in the presence of high concentrations of atropine or gallamine, and then washed and assayed under standard conditions. When the mustard was used alone, almost all of the receptors were alkylated, thus [^{3}H]QNB binding was markedly diminished (Table 2). When atropine (at a concentration 35 times its IC_{50}) was added along with the mustard, good protection from alkylation was afforded, and subsequent [^{3}H]QNB binding was close to the control value. In contrast, gallamine at an equivalent concentration (55 times its IC_{50}) afforded little protection against receptor alkylation by PBCM. Thus most of the [^{3}H]QNB binding sites were alkylated by the mustard

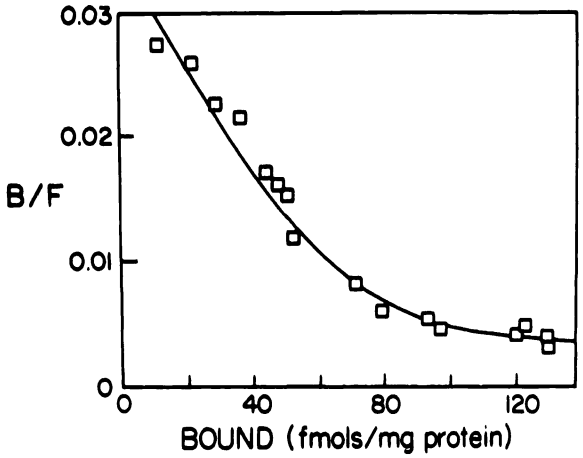

FIG. 5. *Scatchard plot of [^{3}H]QNB binding with pancuronium*
[^{3}H]QNB binding isotherms obtained in the presence of 30 μM pancuronium were best fit by a two-site model with parameter estimates K_1 = 0.033 nM, R_1 = 19.7%; K_2 = 1.57 nM, R_2 = 80.3%. The curve is hand-drawn.

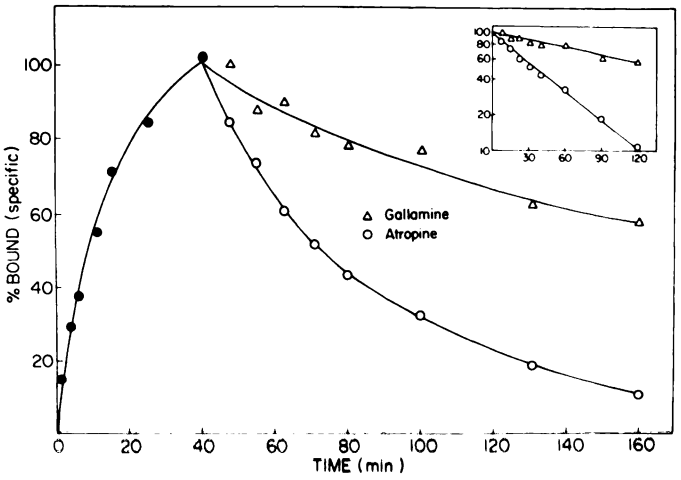

FIG. 6. *Dissociation of [^{3}H]QNB in the presence of excess atropine or gallamine*
Membranes were incubated for 40 min with [^{3}H]QNB (●), and then either 1 μM atropine (○) or 0.5 mM gallamine (△) was added and aliquots were removed for the next 120 min. The *inset* is the log transform of the dissociation curves, from which k_{-1} and $t_{1/2}$ were calculated.

TABLE 2

Atropine but not gallamine protects against muscarinic receptor alkylation

Membranes were incubated with or without 50 nM PBCM, atropine (1 μM), or gallamine (0.3 mM) for 60 min at 30°. The membrane suspensions were then centrifuged, resuspended, and assayed for [^{3}H]QNB binding as described under Materials and Methods except that a saturating concentration of [^{3}H]QNB (0.5 nM) was used. Qualitatively similar results were obtained with a subsaturating concentration of [^{3}H]QNB (0.05 nM) and also when longer or shorter times of incubation with PBCM were tested. Pretreatment with atropine or gallamine in the absence of PBCM did not affect the subsequent binding of [^{3}H]QNB.

Pretreatment[a]	Specific [^{3}H]QNB binding	Receptors alkylated
	cpm	%
No addition	973	
PBCM	104	89
PBCM + atropine	705	28
PBCM + gallamine	203	79

[a] For 60 min at 30°.

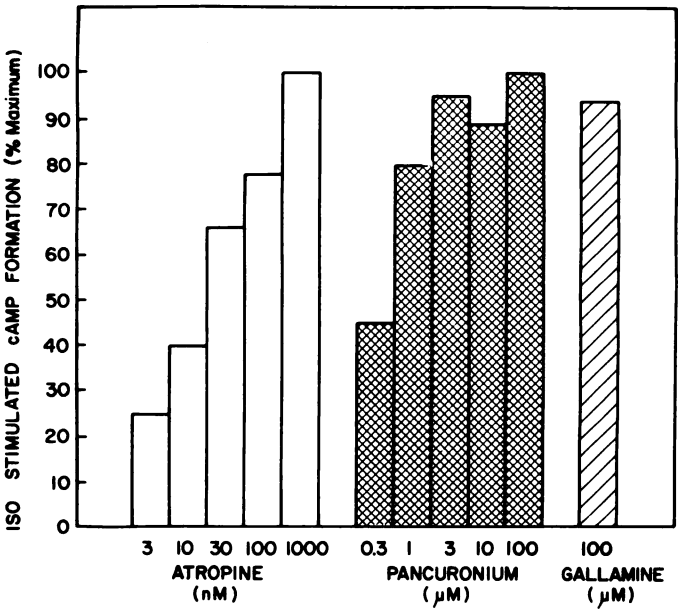

FIG. 7. *Isoproterenol (ISO)-stimulated cyclic AMP formation*
Cyclic AMP concentrations were determined in murine atria treated with 3 μM isoproterenol plus 3 μM carbachol (29% maximal stimulation), or with 3 μM isoproterenol and 3 μM carbachol in the presence of the appropriate concentration of antagonist (data as shown). The data demonstrate the ability of the antagonists to reverse muscarinic inhibition of cyclic AMP accumulation. Each bar represents average values from six atria.

even in the presence of gallamine. As expected, the extent of protection provided by either gallamine or atropine was greatest during a shorter incubation (30 min) and less during a longer incubation (120 min). However, at these times, as at 60 min, gallamine was markedly less protective than atropine.

The unusual behavior of gallamine and pancuronium led us to question whether these agents were truly antagonists of muscarinic receptor-mediated responses. In atria, *beta*-adrenergic receptor stimulation increases cyclic AMP formation, while muscarinic receptor stimulation attenuates this hormonal activation (20). We examined cyclic AMP accumulation in intact atria under conditions in which we previously reported 70–80% inhibition of isoproterenol-stimulated cyclic AMP formation by carbachol (20). The inhibitory effect of carbachol on isoproterenol-stimulated cyclic AMP formation was blocked by atropine in a concentration-dependent manner (Fig. 7). Pancuronium was a surprisingly potent antagonist of muscarinic effects on adenylate cyclase, having an apparent IC_{50} below 1 μM. Furthermore, gallamine completely blocked the receptor-mediated response to carbachol despite the failure of this drug to compete fully for radioligand binding sites.

DISCUSSION

The basic characteristics described here for muscarinic receptors in rat atria are similar to those reported by others (7–9, 21): the binding of [^{3}H]QNB to cardiac muscarinic receptors is described by a simple Langmuir isotherm (Scatchard plots are linear with Hill slopes close to 1), and the K_D from saturation binding experiments is 40–60 pM, consistent with values obtained by kinetic analysis of association and dissociation rates. Classical muscarinic antagonists show the appropriate stereospecificity and compete for [^{3}H]QNB binding with Hill slopes close to 1.

The effects of nicotinic antagonists on cardiac muscarinic receptors have not been previously studied in detail, although Fields *et al.* (7) observed that benzoquinonium was a surprisingly good muscarinic receptor antagonist. We show here that a number of nicotinic antagonists, particularly those with activity directed at neuromuscular sites, compete for [^{3}H]QNB binding at micromolar concentrations. The effects of these agents are atypical in several respects. Unlike muscarinic antagonists, pancuronium, gallamine, and ambenonium compete for [^{3}H]QNB binding sites with Hill slopes less than 1. This apparent heterogeneity in binding is not explained by agonist-like activity of these agents, since all of them antagonize muscarinic receptor-mediated inhibition of cyclic AMP formation. Additionally, since guanine nucleotides do not steepen the nicotinic antagonist competition curves, the observed heterogeneity is apparently unrelated to receptor coupling to a nucleotide binding protein.

The pancuronium competition curve could be fit by a model in which 80% of [^{3}H]QNB binding sites have high affinity and 20% have low affinity for pancuronium. With gallamine, the apparent difference between sites was more extreme, since in this case approximately 20% of [^{3}H]QNB binding was completely insensitive to competition by gallamine at concentrations up to 1 mM. One interpretation of these data is that there are two populations of muscarinic receptors which are distinguished by their differential sensitivity to gallamine and pancuronium, although they are not distinguished by QNB or classical muscarinic antagonists such as atropine. Heterogeneity in [^{3}H]QNB binding results because 80% or so of the receptors are sensitive to gallamine and to low concentrations of pancuronium, and their apparent affinity for [^{3}H]QNB is decreased by these antagonists; the other 20% of the [^{3}H]QNB binding sites are insensitive to gallamine or pancuronium, and the apparent affinity of these sites for [^{3}H]QNB is not changed by these agents.

There are, however, several findings that are difficult to reconcile with this interpretation. First, although approximately 80% of [^{3}H]QNB binding is displaced by gallamine under standard assay conditions, this proportion is decreased in gallamine competition curves obtained at higher [^{3}H]QNB concentrations (Fig. 3). Thus the proportion of [^{3}H]QNB binding sites that appear insensitive to gallamine is not fixed at 20%. Second, if gallamine competitively inhibits [^{3}H]QNB binding to most of the receptor sites, leaving only 20% unaffected, the curve for [^{3}H]QNB dissociation in the presence of gallamine should be displaced only slightly from that seen with atropine. Instead, it is much more shallow, suggesting that [^{3}H]QNB binding is stabilized by the presence of gallamine. Finally, in initial studies on the effects of gallamine in the ileum, we also found approximately 20% of [^{3}H]QNB binding to be gallamine-insensitive. Thus, one would need to postulate that receptor

subtypes distinguished by gallamine exist in the same proportions in atrium and in ileum, a thesis contrary to the physiological data (11–13).

An alternative hypothesis is that receptor heterogeneity is not preexistent but is instead induced by the binding of gallamine or pancuronium. If gallamine or pancuronium binds at a second site on the receptor, these agents could allosterically affect [^{3}H]QNB binding at the primary site. The formation of a ternary complex in which the receptor simultaneously binds gallamine and [^{3}H]QNB is consistent with several of our observations. For example, gallamine did not effectively protect receptors against alkylation by PBCM (Table 2), suggesting that the binding of gallamine does not exclude binding of PBCM to muscarinic receptors. Additionally, the dissociation of [^{3}H]QNB from the receptor was slowed in the presence of gallamine, an observation consistent with an allosteric effect of gallamine on the kinetics of [^{3}H]QNB binding at an adjacent site. Finally, the proportion of [^{3}H]QNB binding that is not inhibitable by gallamine increases as the [^{3}H]QNB concentration is raised (Fig. 3). One explanation for this is that more receptor is in a state binding both [^{3}H]QNB and gallamine, a state in which QNB does not readily dissociate from the receptor. The possibility of a ternary complex between the muscarinic receptor, [^{3}H]*N*-methylscopolamine, and gallamine was also suggested in a recent abstract by Birdsall *et al.* (26).

Thus several lines of evidence support the hypothesis that gallamine binds at a secondary site on the muscarinic receptor and influences the kinetics of [^{3}H]QNB binding. However, this allosteric mechanism would not necessarily lead to heterogeneity in [^{3}H]QNB binding isotherms. We therefore suggest that there is also a competitive interaction between gallamine and [^{3}H]QNB at the primary binding site. Such an interaction is consistent with the competitive nature of the Scatchard plots at low concentrations of gallamine (Fig. 4) and is also consistent with the finding that the K_i for gallamine is the same at all [^{3}H]QNB concentrations (Fig. 3). Clark and Mitchelson (27) and Lullman *et al.* (28) found that blockade of the inotropic effects of ACh by gallamine or BTM derivatives appeared competitive, as characterized by parallel rightward shifts in the agonist dose-response curves. However, since the effectiveness of these antagonists diminished at high concentrations and the combination of these agents with atropine was not additive, they also suggested an allosteric effect on the cardiac muscarinic receptor.

Normally, [^{3}H]QNB appears to bind to identical and noninteracting sites on cardiac muscarinic receptors. Under conditions of low ionic strength (10) or in the presence of choline or Tris (29), heterogeneity in antagonist binding to the cardiac muscarinic receptor is induced through as yet unexplained mechanisms. In our studies, a pharmacological intervention, i.e., the presence of gallamine or pancuronium, also induces heterogeneity in antagonist binding. We suggest that gallamine exerts this effect by binding at a secondary binding site on the cardiac muscarinic receptor in addition to acting as a competitive ligand at the [^{3}H]QNB binding site. The structural or functional relationship between primary and secondary ligand binding sites on the muscarinic receptor is not clear. However, it may be the distance between these sites and the binding determinants on these sites that confers muscarinic receptor activity upon certain cholinesterase inhibitors and nicotinic antagonists with bis- or tris-quaternary structures.

Our studies with gallamine and other nicotinic antagonists were motivated by reports that these agents selectively block certain muscarinic responses in heart and ganglia (3, 11–15). Our preliminary data suggested that gallamine and pancuronium might have different affinities for subtypes of cardiac muscarinic receptor (16), a conclusion recently suggested by Ellis and Hoss (30) in a paper describing effects of gallamine on brain muscarinic receptors. The data presented here suggest that receptor heterogeneity is induced by the actions of gallamine and pancuronium, rather than detected by them. Tissue specificity or receptor selectivity might be explained by differences in the extent to which gallamine binds competitively versus noncompetitively, and by the ability of gallamine to affect allosterically the primary receptor binding site. Further studies with agents such as gallamine and pancuronium should help to elucidate the structure and regulation of muscarinic receptors, and may demonstrate that the subclassification of muscarinic receptors requires characterization of secondary as well as primary ligand binding sites on the muscarinic receptor.

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