could cause the reduced binding. In imipramine a ring nitrogen replaces the carbon found in amitriptyline and a double bond, which is present in folic acid, is lost. These changes could account for the increase in K_i values.

Thus a new class of compounds, the tricyclic antidepressant drugs, cause significant inhibition of dihydrofolate reductase. In considering the practical implications of this finding it should be borne in mind that the K_i values are more than 1000-fold greater than that for methotrexate while the therapeutic blood levels used for the tricyclic drugs are about 100-fold lower than those used for methotrexate. Nevertheless, it is possible that long term treatment with the tricyclic antidepressant drugs could lead to their accumulation in tissues—the consequences of this in terms of exacerbation of folate deficiency require further consideration.

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Characterization of the interactions of gallamine with muscarinic receptors from brain

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A previous report by Ellis and Hoss [1] demonstrated that gallamine inhibits the binding of the potent and specific muscarinic antagonist quinuclidinyl benzilate (QNB). Although other studies have found gallamine to be an antagonist in muscarinic systems [2–5], the binding properties of gallamine were shown to be similar to those of muscarinic agonists. That is, the occupancy curve for gallamine is better suited to a model of two populations of receptors than to a one-site model and it possesses greater overall affinity toward brainstem receptors than toward those of the forebrain [1]. Recently, Stockton *et al.* [6] reported that gallamine regulates muscarinic receptors by an allosteric mechanism. The possibility was therefore raised that the heterogeneity detected by gallamine in the earlier study might have been artifactual, due to the assumption of an incorrect mechanism of action. However, it is difficult to account for all of the previous findings of Ellis and Hoss by a noncompetitive mechanism, especially that the elimination of receptors with low affinity for the agonist carbachol results in a loss of receptors with low affinity for gallamine [1]. The present study was undertaken in an attempt to reconcile these apparently discrepant reports and to further evaluate the mechanism(s) by which gallamine interacts with muscarinic receptors.

Tritiated L-QNB (32.2 Ci/mmole) and [^{3}H]*N*-methylscopolamine (NMS, 84.8 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Gallamine triethiodide was obtained from K & K Laboratories (Plainsview, NY). Neural membranes were prepared from the forebrains of male Sprague–Dawley rats as described previously [1] and stored frozen at −70° until used. The term forebrain denotes the portion of the brain anterior to the diencephalon, plus overlying cortical and hippocampal tissue. Binding assays were conducted at 25° in 40 mM sodium–potassium phosphate buffer (except where otherwise indicated), pH 7.2, and were terminated by filtration through GF/B glass fiber filters (Brandel, Gaithersburg, MD). In all cases, nonspecific binding was determined by the inclusion of 1 μM unlabeled QNB with the labeled ligand. Under conditions of low ionic strength, high concentrations of gallamine were found to decrease the nonspecific binding of [^{3}H]QNB. Therefore, nonspecific binding was determined for each concentration of gallamine by including 1 μM unlabeled QNB with the labeled QNB and gallamine in a separate set of assay tubes.

The results of experiments which employed [^{3}H]NMS (Figs. 1A and 2A) were consistent with the report by Stockton *et al.* [6]. Both the association and dissociation kinetics were dramatically slowed by the presence of gallamine. Separate studies (data not shown) found the half-maximal effect on the off-rate of [^{3}H]NMS to occur at 30 μM gallamine. However, when similar studies were carried out with [^{3}H]QNB (Figs. 1B, 1C, and 2B), no allosteric effects were noted. Even at 100 μM, gallamine failed to significantly alter the rate of association of [^{3}H]QNB or the rate of dissociation of previously bound [^{3}H]QNB.

The next question that we wished to address was whether the muscarinic sites that appear to possess different affinities for gallamine are separate and non-interconvertible. One approach to this question is to selectively block the low-affinity sites with an irreversible or slowly reversible antagonist (e.g. QNB), as has been done in the past for the sites that are differentiated by the muscarinic agonists [1, 8, 9]. Initial studies (Fig. 3) found that the different sites expressed the most widely disparate affinities for gallamine under conditions of low ionic strength. Such conditions were therefore chosen for the protection experiments. Forebrain membranes were preincubated in the presence of unlabeled QNB and a concentration of gallamine that would compete efficiently only at gallamine's high-affinity sites. Extensive washing then removed bound and unbound gallamine and unbound QNB; more than 90% of the bound

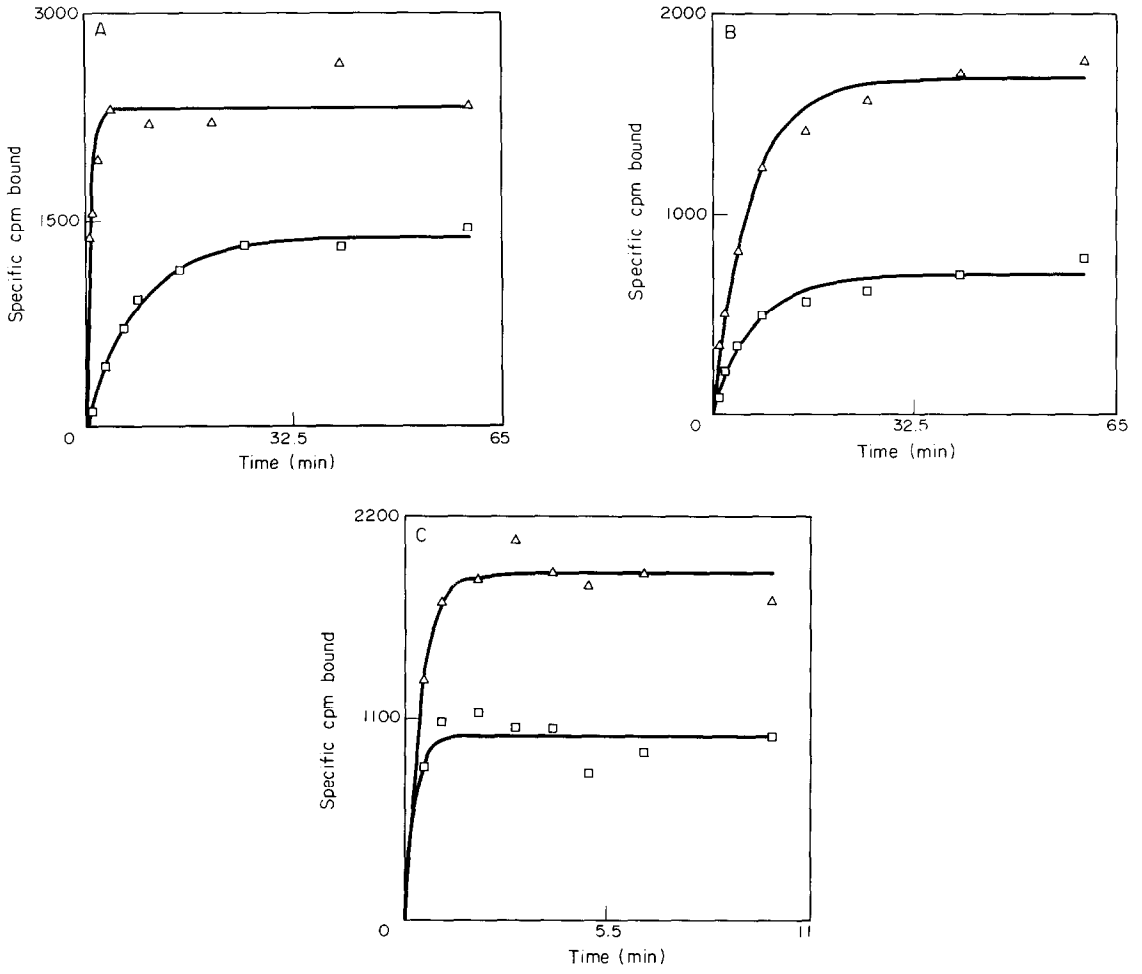

Fig. 1. Time course of inhibition by gallamine of muscarinic receptor binding. Labeled NMS (A, 1 nM) or QNB (B, 0.5 nM; C, 2.5 nM) was incubated with forebrain membranes for the indicated times in the absence (△) or presence (□) of 3 μM (A), 15 μM (B), or 100 μM (C) gallamine. The receptor concentration was 40 pM, representing approximately 0.06 mg protein in a volume of 2 ml. The solid lines indicate monoexponential best-fits to the data, which were carried out through the use of the non-linear curve-fitting software MLAB, which is freely available from NIH [7]. The derived values for the half-times (in minutes) to approach equilibrium in the absence and presence of gallamine, respectively, were: A, 0.52, 4.6; B, 3.9, 4.5 and C, 0.28, 0.17.

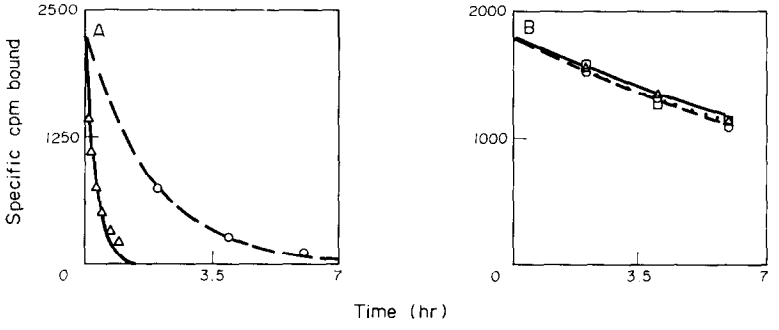

Fig. 2. Effect of gallamine on dissociation of labeled ligands from the muscarinic receptor. Labeled NMS (A, 1 nM) or QNB (B, 1 nM) was allowed to equilibrate for 30 min with muscarinic receptors of forebrain membranes (receptor concentration, 400 pM). At time $t = 0$, 0.25 ml of the labeled membranes was diluted 8-fold in an excess of unlabeled QNB (1 μM), without (△) or with 15 μM (□) or 100 μM (○) gallamine. As in Fig. 1, the curves represent monoexponential best-fits to the data. The times required for dissociation of 50% of the bound labeled ligands were: (A) no gallamine, 12.2 min; 100 μM gallamine, 87 min: and (B) no gallamine, 10.8 hr; 15 μM gallamine, 11.3 hr; 100 μM gallamine, 11.7 hr.

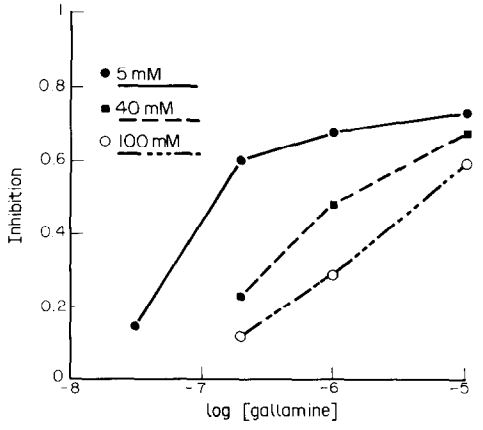

Fig. 3. Effect of ionic strength on the potency of gallamine. Forebrain membranes were incubated for 90 min with 0.1 nM [^{3}H]QNB and the indicated concentrations of gallamine in 5 mM, 40 mM, or 100 mM sodium potassium phosphate buffer, pH 7.2. The receptor concentration was 6 pM in a volume of 10 ml. The results are expressed as the fractional inhibition by gallamine of the specific binding of [^{3}H]QNB.

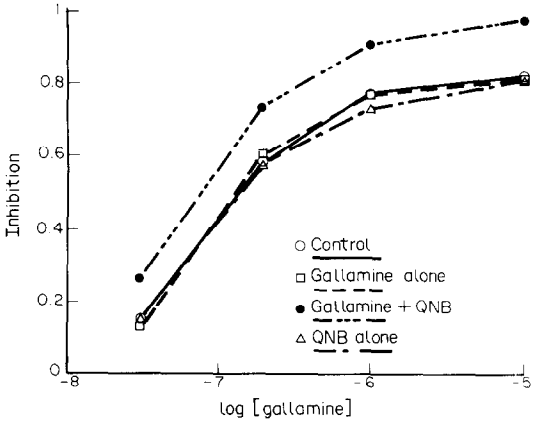

Fig. 4. Selective protection of sites with high affinity for gallamine. Forebrain membranes were preincubated in 5 mM sodium–potassium phosphate buffer for 15 min at 25° under one of the following conditions: (a) no addition; (b) 1 μM gallamine; (c) 2 nM unlabeled QNB; and (d) 1 μM gallamine plus 10 nM unlabeled QNB. The membranes were then sedimented, washed twice to remove the unbound QNB and the bound and unbound gallamine, and resuspended. The number of sites remaining per mg protein was determined after each preincubation and, as a percentage of the control value (a), are as follows: (b) gallamine alone, 104%, (c) QNB alone, 42%; (d) gallamine plus QNB, 72%. These membranes were also assayed in 5 mM phosphate buffer to determine the potency of gallamine in inhibiting the binding of [^{3}H]QNB to the sites that remained, as in Fig. 3. The curves shown are representative of three separate experiments. When analysis of variance was carried out on the cumulated data from the three experiments, a significant effect of preincubation condition was confirmed [$F(3, 32) = 6.40$, $P < 0.01$]. Subsequent evaluation by Duncan's multiple-range test found that preincubation with gallamine plus QNB (condition d) differed significantly from all other conditions ($P < 0.005$) but that conditions a–c did not differ significantly from each other.

QNB would be expected to remain on the receptors for the duration of the experiment. Subsequent assays of membranes that had been preincubated in this manner showed that the sites with low affinity for gallamine had been selectively and dramatically blocked by the unlabeled QNB (Fig. 4). However, preincubation in the presence of gallamine alone or QNB alone either had no effect on the subsequent assay or reduced the number of receptors without regard to affinity toward gallamine respectively (Fig. 4).

It is clear that the binding of [^{3}H]NMS is subject to noncompetitive regulation by gallamine (Figs. 1A and 2A). At the same time, data obtained by the use of [^{3}H]QNB (Figs. 1B, 1C and 2B) strongly indicate that gallamine interacts competitively with the muscarinic receptor, supporting the conclusions of our previously reported study in which [^{3}H]-QNB was employed as the labeled ligand [1]. The fact that sites with low affinity for gallamine can be selectively eliminated (Fig. 4) confirms that there are separate, noninterconvertible, subpopulations with regard to affinity toward gallamine.

One possible explanation for the data presented in this article is that gallamine binds to an allosteric site as well as to the site to which QNB binds. According to this scheme, QNB must be insensitive to the allosteric interaction that affects the binding of NMS. Another possibility is that QNB and gallamine interact allosterically, but that the binding of either ligand reduces the affinity of the other to such an extent that ternary complexes cannot be demonstrated. This type of interaction is indistinguishable from a competitive inhibition. Even this hypothesis, however, requires the additional assumption of some form of heterogeneity in the binding of gallamine to explain the data shown in Fig. 4. While it is somewhat surprising that such different conclusions can be reached through the use of [^{3}H]QNB and [^{3}H]NMS, it is possible that the positively charged NMS might interact with the receptor in a different manner than does the very lipophilic QNB. It remains to be seen whether most muscarinic ligands will be found to behave like [^{3}H]NMS or like [^{3}H]QNB in the presence of gallamine.

There are, as yet, relatively few antagonists that discriminate subpopulations of muscarinic receptors. There are fewer still that divide muscarinic receptors into subpopulations that are known to have some relationship to the subpopulations that are discriminated by agonists [10], as has been indicated to be true for gallamine [1]. Therefore, we expect gallamine to continue to be useful in the characterization of subpopulations of muscarinic receptors.

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Effect of methyl palmoxirate, an oral hypoglycemic agent, on epinephrine-induced hyperglycemia in the rat

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Methyl palmoxirate (methyl 2-tetradecylglycidate), a potent specific inhibitor [1] of carnitine palmitoyl transferase (CPT), has been shown to be a hypoglycemic and hypoketonemic agent in diabetic animals [2–4] and man [5]. Evidence has been presented that the lowering of blood glucose results from inhibition of hepatic gluconeogenesis [2, 6, 7] and from stimulation of peripheral glucose utilization [2, 8, 9]. Since there is increasing evidence that the long-term complications of diabetes are related in part to poor glucose control [10, 11], there has been increasing effort to reduce the elevated blood glucose levels as close to the normal range as possible [12–14], but this approach may increase the risk of severe hypoglycemia [15]. Obviously, for any new oral hypoglycemic agent, such as methyl palmoxirate (MP), a similar concern exists. Counterregulatory hormones are known to play a critical role in defense against hypoglycemia. Therefore, it was of interest to determine whether a counterregulatory hormone, such as epinephrine, could elevate blood glucose in fed or fasted rats that had been treated with methyl palmoxirate.

Methods

Male Sprague–Dawley rats (200–280 g) from Charles River, maintained on Lab-Blox rat chow (Wayne), were used throughout. When required, they were fasted 18–24 hr prior to the epinephrine treatment.

Animals were dosed daily with MP or vehicle (0.5% methylcellulose). On the day of the study, all rats were bled from the tail vein for blood glucose determination, dosed, and 3 or 4 hr post dosing blood samples were again taken for measurement of glucose changes prior to and at 30, 60 and 90 min after administration of epinephrine bitartrate (Sigma Chemical Co., St. Louis, MO). Blood glucose was determined using Autoflo Glucose (Bio-Dynamics/bmc, Indianapolis, IN). In some experiments, animals were killed by decapitation immediately following the final blood sampling; heart, liver and gastrocnemii were removed for determination of glycogen [16]; and plasma samples were collected for analysis of free fatty acids (FFA) [16, 17]. In some cases, mitochondrial carnitine palmitoyl transferase was measured [1, 2] using a piece of the liver. The 3-mercaptopicolinic acid was obtained as a gift from Smith Kline & French Laboratories (Philadelphia, PA). 2,5-anhydro-*d*-mannitol was prepared at McNeil Pharmaceutical by Allen B. Reitz, and 5-methoxyindole-2-carboxylic acid was purchased from the Aldrich Chemical Co. (Milwaukee, WI).

For *in vitro* studies, hepatocytes from 24-hr fasted rats were isolated and incubated (30 min) under conditions reported elsewhere [6].

Results and discussion

As shown in Fig. 1, a dose of 83 μg/kg, s.c., of epinephrine produced a highly significant ($P < 0.01$) hyperglycemic response (approximately 100% increase of blood glucose), using either fed or fasted rats. As shown in Fig. 1, 3 days of once-a-day treatment with a therapeutic dose [2, 5] of MP (1.0 mg/kg, p.o.) did not diminish significantly this hyperglycemic effect of epinephrine. This dose of MP has been reported to lower plasma ketones and to inhibit liver mitochondrial CPT activity by >90% in fasted rats [18] and to increase plasma FFA levels [2, 3]. In the present study, the plasma FFA of the fasted rats increased 89% following MP treatment though, as reported previously [2, 3], MP did not elevate plasma FFA or lower blood glucose in fed rats (results not shown).

The dose of MP was then increased to produce near maximal decreases of plasma glucose and liver glycogen levels of fasted rats. With such a high dose, it was expected that a greater effect on the epinephrine response would be seen in the fasted state than in the nonfasted state, and this was found to be the case. In fasted rats where MP (2.5 mg/kg p.o./day for 3 days) lowered the baseline blood glucose 30% (Fig. 2) and decreased the heart and liver but not skeletal muscle glycogen levels (see inset), some significant, though small, attenuation of the epinephrine response was observed (Fig. 2). Using *ad lib.* fed rats, this dose of MP failed to significantly reduce liver or muscle glycogen levels or the hyperglycemic effect of epinephrine (results not shown).

Shikama and Ui [19], using intraperitoneally administered epinephrine, found enhanced incorporation of radioactively labeled gluconeogenic precursors into both blood glucose and liver glycogen of fasted nondiabetic rats. Other investigators have also reported [18, 20] increased deposition of liver glycogen in fasted rats following epinephrine injection. We also found that intraperitoneal administration of a high dose of epinephrine (0.2 mg/kg) to fasted rats resulted in a quantitatively small (compared to fed state) though significant increase in liver glycogen without altering the heart or skeletal muscle glycogen (results not shown). This epinephrine-induced increase of liver glycogen (level increased from 0.5 ± 0.6 in the controls to 4.2 ± 1.2 mg glucose/g liver at 90 min after epinephrine) was totally suppressed by 10 days of treatment with MP (2.5 and 5.0 mg/kg/day) even though the hyperglycemic effect of epinephrine was only partially inhibited. As