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ALLOSTERIC REGULATION OF CLONED m1–m5 MUSCARINIC RECEPTOR SUBTYPES

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Abstract—Allosteric regulation of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) and [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) dissociation from the m1–m5 muscarinic receptor subtypes was examined in transfected CHO-K1 cells. Half-times of dissociation of [^{3}H]NMS from cell membranes (at 23°) ranged from less than 5 min for the m2 subtype to more than 60 min for the m5 subtype. For [^{3}H]QNB, half-times (at 37°) ranged from 1 hr (m2) to almost 4 hr (m3). The presence of gallamine slowed the dissociation of [^{3}H]NMS from all of the subtypes, with an order of potency of m2 > m4 > m1 > m3 > m5. Dissociation of [^{3}H]QNB from m1 and m2 receptors was modulated by gallamine in the biphasic manner that we have described previously for cardiac receptors; that is, low concentrations (1–10 μM) of gallamine accelerated dissociation, while 1 mM gallamine slowed it. Verapamil slowed the dissociation of [^{3}H]-QNB from the m2 receptor in a monophasic manner, while the action of *d*-tubocurarine was qualitatively similar to that of gallamine. The potency of gallamine in allosterically regulating the m2 receptor was inversely related to ionic strength. Inactivation of pertussis toxin-sensitive G proteins abolished the ability of guanine nucleotides to regulate agonist affinity at the m2 receptor, but had no effect on allosteric regulation of the m2 receptor. These findings indicate that susceptibility to allosteric regulation varies in a complex way across muscarinic receptor subtypes and according to the choice of ligand.

Muscarinic antagonists such as atropine and scopolamine interact with muscarinic agonists in a competitive manner [1]. However, many studies have shown that muscarinic receptors are also susceptible to allosteric modulation by a pharmacologically diverse array of compounds [2–4]. One of these, gallamine, has been shown to produce biphasic allosteric effects on the binding of [^{3}H]-quinuclidinyl benzilate ([^{3}H]QNB‖) to muscarinic receptors from heart and brain, suggesting the existence of multiple allosteric sites for gallamine [5]. Five muscarinic receptor subtypes have been identified by molecular cloning studies and are selectively expressed in tissue-specific patterns [6–9]. It has been suggested that some allosteric modulators may exhibit subtype-selectivity [5, 10, 11]. However, evaluation of this supposition may be complicated by the co-expression of multiple muscarinic subtypes in many tissues [7–9], the use of different ligands to label different receptor subtypes, or possible tissue-specific differences in receptor folding [1]. For this reason, receptor subtypes would ideally be studied in isolation, but in the same tissue. In that way they could each be labeled by the same ligand, and tissue-specific effects would be avoided. In the present study, we examined allosteric modulation of [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS) and [^{3}H]QNB binding to all five muscarinic receptor subtypes, expressed in CHO-K1 cells. We found that all of the subtypes are susceptible to allosteric regulation; the characteristics of this regulation are unique for each subtype, but also depend on the choices of ligands at both competitive and allosteric sites.

MATERIALS AND METHODS

Tritiated l-QNB (35.2 Ci/mmol) and [^{3}H]NMS were purchased from NEN (Dupont; Boston, MA). Atropine, gallamine, verapamil, *d*-tubocurarine, and pertussis toxin were obtained from the Sigma Chemical Co. (St. Louis, MO). Cell culture materials were from GIBCO Laboratories (Grand Island, NY). Glass fiber filters (SS #32) were from Schleicher & Schuell (Keene, NH).

CHO-K1 cells that had been stably transfected with the genes for single muscarinic receptor subtypes [12] were grown in F12 medium supplemented with 10% serum to 60–75% confluency. The receptor subtypes investigated were the rat m1 subtype and the human m2, m3, m4, and m5 subtypes [12]. Cells were harvested by scraping into 5 mM sodium-potassium phosphate buffer (PB), pH 7.4, and homogenized in a motorized glass–teflon homogenizer. Membranes were pelleted at 30,000 *g* for 25 min, resuspended in 5 mM PB, and stored frozen at −70°.

Muscarinic allosteric interactions have been inferred from a variety of techniques. Some studies have analyzed functional responses with pharmacological null methods, such as Schild plots,

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‖ Abbreviations: QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; PB, sodium-potassium phosphate buffer; and m1–m5, the designations of cloned muscarinic receptor subtypes (see Ref. 24).

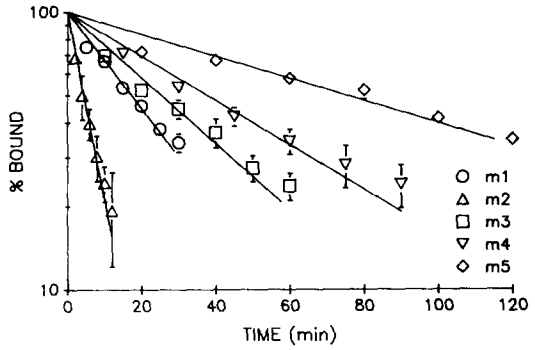

Fig. 1. Dissociation of [^{3}H]NMS from m1–m5 subtypes. Membranes from CHO-K1 cells that had been transfected with the appropriate muscarinic receptor subtype were prelabeled with [^{3}H]NMS as described in Materials and Methods. At time t = 0, atropine was added and [^{3}H]NMS dissociated from the subtypes as indicated, at 22° in 5 mM phosphate buffer. Data points are the means ± SEM from 3–4 experiments.

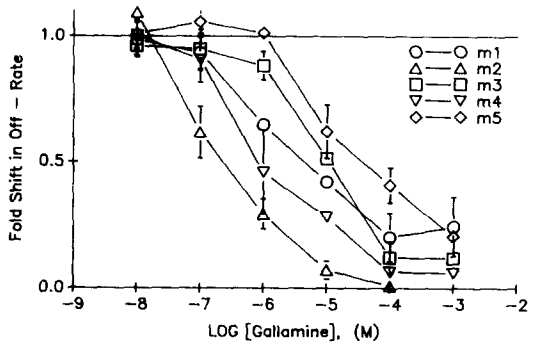

Fig. 2. Modulation by gallamine of the dissociation kinetics of [^{3}H]NMS at m1–m5 subtypes. Experiments were conducted as in Fig. 1, except that the indicated concentration of gallamine was present during the dissociation phase. Results are expressed as the apparent k_{off} in the presence of the indicated concentrations of gallamine divided by k_{off} in the absence of gallamine (see Materials and Methods). Results are the means ± SEM of 3–4 experiments.

dose-ratio additivity, or resultant analysis [1, 13, 14]. Others have analyzed binding assays at or near equilibrium according to equations of allosteric interaction [15, 16]. Such approaches have clearly demonstrated the existence of allosteric interactions, but cannot rule out the possible contribution of concomitant competitive interactions. There is good reason to believe that those allosteric agents that have been most closely investigated act *both* competitively and allosterically [14, 17, 18]; indeed, the existing data indicate that the competitive interaction is more potent [3, 19, 20]. In the present study, we investigated the allosteric modulation of dissociation kinetics, a technique which is not affected by concomitant competitive interactions [17, 18]. These dissociation studies were conducted by allowing labeled ligands (1 nM) to equilibrate with membranes (0.02 to 0.1 mg protein per tube) for 30 min at 22° ([^{3}H]NMS) or 60 min at 37° ([^{3}H]-QNB). This resulted in specific binding at time t = 0 of 4,000–10,000 dpm in these experiments. Dissociation was initiated by the addition of atropine (1 μM final concentration), with or without allosteric modifiers. The incubation was terminated at the appropriate time by filtration through SS #32 glass fiber filters.

Dissociation data were fitted to a monoexponential function to obtain the best-fit rate constants (k_{off}), and then displayed in the figures as a logarithmic transform. In experiments on the concentration-dependence of allosteric modifiers, the off-rates of the labeled ligands were determined in the presence and absence of the allosteric modifiers. The ratio of these two values was designated the fold-shift in the off-rate; fold-shift values greater than one indicate an increase in the rate of dissociation (decrease in half-time).

RESULTS

The dissociation rate constants for [^{3}H]NMS were found to vary widely across receptor subtypes (Fig. 1): m2 (0.155 min^{-1}) > m1 (0.04) > m3 (0.0273) > m4 (0.0184) > m5 (0.00924). Thus, half-times of dissociation ranged from less than 5 min (m2) to more than 1 hr (m5). The dissociation of [^{3}H]QNB was much slower. Even at 37°, the most rapidly dissociating subtype (m2) displayed a half-time of about 1 hr, the slowest (m3) almost 4 hr. Under these conditions, the dissociation rate constants for [^{3}H]QNB (data not shown) were: m2 (0.0105 min^{-1}) > m4 (0.0079) > m5 (0.0064) > m1 (0.00457) > m3 (0.00315). The presence of gallamine produced a concentration-dependent slowing of dissociation of [^{3}H]NMS at all subtypes, with an order of potency of m2 > m4 > m1 > m3 > m5 (Fig. 2). The concentration of gallamine required to slow the dissociation of [^{3}H]NMS by 50% varied from about 0.2 μM at the m2 subtype to about 30 μM at the m5 subtype. The interactions between gallamine and [^{3}H]QNB were more complex. As previously reported for cardiac muscarinic receptors [5], gallamine produced a biphasic effect at the m2 subtype. At a low concentration (1 μM), gallamine accelerated the dissociation of [^{3}H]QNB, while at a higher concentration (1 mM) the dissociation was slowed (Fig. 3). The concentration-dependency is more fully characterized in Fig. 4, where it can be seen that the two effects at the m2 subtype cancelled each other at about 100 μM gallamine, yielding no net change in the rate of dissociation. The m1 subtype also exhibited a marked acceleration of dissociation. The other subtype appeared to follow the same biphasic trend, but with smaller magnitudes of both effects (Fig. 4).

The potency with which gallamine slowed the rate of dissociation of [^{3}H]NMS from the m2 subtype was very sensitive to the ionic strength of the buffer. As the phosphate buffer concentration rose from 5 to 50 mM, approximately 100-fold higher concentrations of gallamine were required to produce equal reductions in the rate of dissociation (Fig. 5). The effect of ionic strength on the modulation by

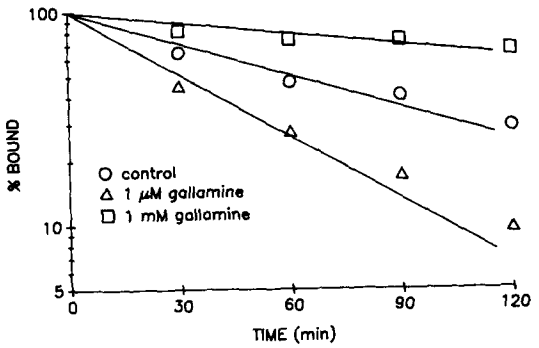

Fig. 3. Dissociation of [^{3}H]QNB from the m2 subtype in the presence of low and high concentrations of gallamine. Membranes from CHO-KI cells transfected with m2 receptors were prelabeled with [^{3}H]QNB as described in Materials and Methods. The dissociation of [^{3}H]QNB at 37° was accelerated by 1 μM gallamine (k_{off} = 0.0224 min^{-1}) and slowed by 1 mM gallamine (0.004), relative to control (no gallamine; 0.0114). Results are the means of 2 experiments.

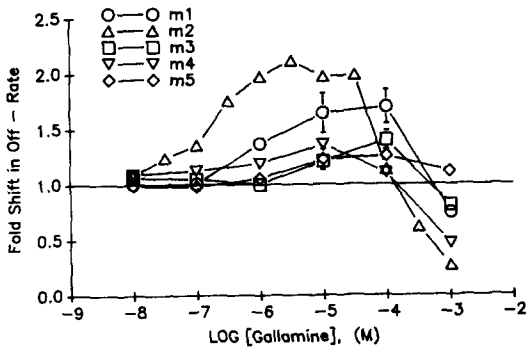

Fig. 4. Modulation by gallamine of the dissociation kinetics of [^{3}H]QNB at m1–m5 subtypes. Experiments were conducted as in Fig. 3 and results are expressed as in Fig. 2, as the means ± SEM of 3–5 experiments.

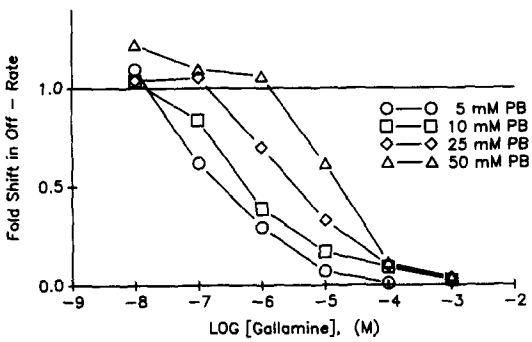

Fig. 5. Effect of ionic strength on the modulation by gallamine of [^{3}H]NMS dissociation from m2 sites. Experiments were conducted as in Fig. 2, except that only m2 sites were examined and the buffer concentration was varied as indicated. Each curve represents the mean of 2–3 experiments.

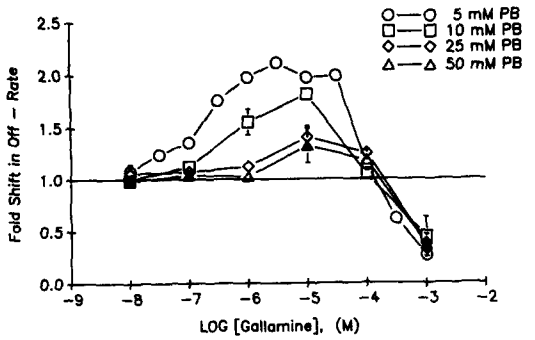

Fig. 6. Effect of ionic strength on the modulation by gallamine of [^{3}H]QNB dissociation from m2 sites. As for [^{3}H]NMS dissociation (Fig. 5), the potency of gallamine was evaluated at varying ionic strengths. Results are the means ± SEM of 3–5 experiments.

gallamine of the dissociation of [^{3}H]QNB was, not surprisingly, more complex (Fig. 6). It appeared that the acceleratory component was shifted to the right (toward higher gallamine concentrations), in much the same manner as was the curve for [^{3}H]NMS in Fig. 5. However, the magnitude of this acceleratory effect was reduced sharply at 25 and 50 mM phosphate buffer, possibly due to interaction with the second phase (deceleratory) effect, which seemed to be unaffected by the change in ionic strength (Fig. 6).

Two other allosteric modulators, verapamil and *d*-tubocurarine, were examined at the m2 and m3 subtypes. Verapamil was about 10-fold less potent than *d*-tubocurarine in slowing the dissociation of [^{3}H]NMS from both receptors (Fig. 7, top and middle panels). Both agents were about 10-fold more potent at the m2 subtype (Fig. 7, middle panel) than at the m3 subtype (Fig. 7, top panel). Neither modulator was particularly effective at accelerating the dissociation of [^{3}H]QNB from the m2 subtype, but *d*-tubocurarine was about 10-fold *less* potent than verapamil at slowing the dissociation of [^{3}H]-QNB from that subtype (Fig. 7, bottom panel).

We chose to investigate possible G-protein involvement in the allosteric regulation of the m2 subtype, because this subtype exhibited the most potent and complex effects of gallamine (Figs. 2 and 4) and because it couples to pertussis toxin-sensitive proteins [21]. Treatment of cells that had been transfected with the m2 subtype with 0.1 μg/mL pertussis toxin (in culture, overnight) completely abolished the GTP shift in agonist affinity that was seen in membranes derived from control cells (data not shown). However, the allosteric effects of gallamine on [^{3}H]NMS and [^{3}H]QNB dissociation from the same membranes were unaffected by the toxin treatment (Fig. 8).

DISCUSSION

The present experiments have confirmed earlier suggestions that the allosteric effects of gallamine are subtype-selective [5, 10, 11, 19] and that the choice of ligand markedly influences the potency

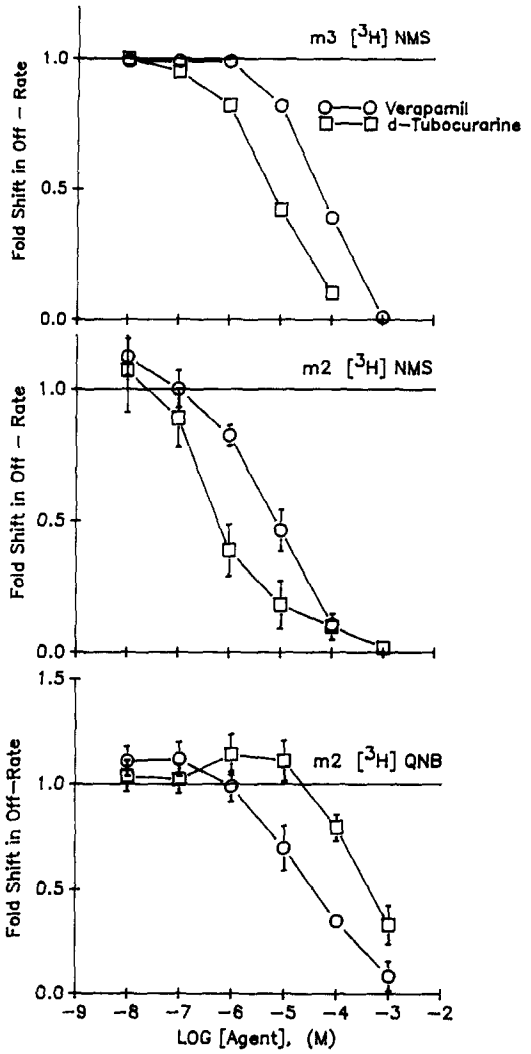

Fig. 7. Modulation of the dissociation of [^{3}H]NMS and [^{3}H]QNB by verapamil and *d*-tubocurarine. Experiments were conducted as in Figs. 2 and 4, except that verapamil or *d*-tubocurarine were present during the dissociation phase. Top panel, dissociation of [^{3}H]NMS from m3 receptors (*means of 2 experiments*); *middle, dissociation* of [^{3}H]NMS from m2 receptors (means ± SEM of 3–4 experiments); bottom, dissociation of [^{3}H]QNB from m2 receptors (means ± SEM of 3 experiments).

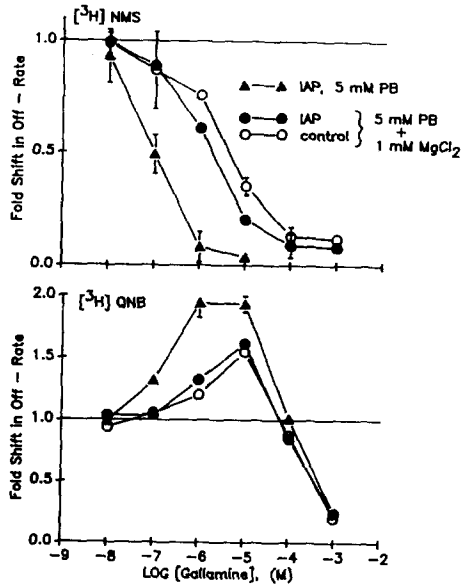

Fig. 8. Modulation of the dissociation of [^{3}H]NMS and [^{3}H]QNB from m2 sites after G-protein inactivation by pertussis toxin. Cells were treated with 0.1 μg/mL pertussis toxin (IAP) overnight. The effectiveness of the IAP treatment was confirmed by demonstrating that the GTP-shift in agonist affinity (carbachol) was abolished (data not shown). Assays were conducted in 5 mM phosphate buffer (as in previous figures) or in 5 mM phosphate buffer with 1 mM $MgCl_2$ (the buffer used in the carbachol GTP-shift assays). Results are the means ± SEM of 3 experiments.

and extent of the allosteric effects (compare Figs. 2 and 4). These findings emphasize the need to compare known subtypes labeled with the same ligand if meaningful comparisons are to be drawn. For example, Michel *et al.* [11] reported that gallamine modulates the rate of dissociation of [^{3}H]-NMS from glandular ("M_3") receptors at much lower concentrations than were needed to slow the dissociation of [^{3}H]pirenzepine from cortical ("M_1") receptors. However, exocrine glands have been shown to contain mRNA for both the m1 and m3 subtypes [9], and [^{3}H]pirenzepine labels m4 as well as m1 in rat cortex [8]. Using CHO cells that express specific subtypes of the receptor, we were able to label each site, in isolation, with [^{3}H]NMS. Under our conditions, gallamine slowed the dissociation of [^{3}H]NMS slightly more potently at m1 receptors, compared to m3 receptors, consistent with another recent report that used transfected CHO cells [19]. Michel *et al.* also found that gallamine and methoctramine induced a dramatic non-muscarinic binding of [^{3}H]NMS. That is, when submaxillary gland membranes were prelabeled with [^{3}H]NMS, the presence of gallamine or methoctramine ($\geq 10^{-4}$ M) over the subsequent 30 min *increased* the binding of [^{3}H]NMS, even though an excess of unlabeled atropine (1 μM) was present during that 30-min interval [11]. It is not clear whether a possible heterogeneity of subtypes in the assay (see above) may have contributed to this phenomenon, but we did not observe any such complications with the CHO cell membranes.

We found marked differences between the rates of dissociation of [^{3}H]NMS from the subtypes. The rates of dissociation agree reasonably well with a previous report by Waelbroeck *et al.* [22], in which differences in dissociation rates were correlated with pharmacological characteristics. Thus, their "C" (cardiac) receptor was the fastest, comparable to our m2; their "A" receptor was intermediate in dissociation rate and displayed high affinity for pirenzepine, comparable to our m1; their "B" receptor was the most slowly dissociating component from rat forebrain, and displayed intermediate affinity for pirenzepine—this component could

represent a mixture of m3 and m4 subtypes, which would be difficult to distinguish either by off-rate (Fig. 2) or affinity for pirenzepine [23, 24]. Indeed, in a more recent paper, these authors have suggested that the B component is composed of two subtypes [20]. The most slowly dissociating component, m5, is less prominently expressed [8], although *in situ* hybridization studies have found a specific labeling of neurons of the substantia nigra, pars compacta [7].

The rate of dissociation of [^{3}H]QNB varied less across subtypes, and with a different order (m1 and m3 were slower than m4 and m5). It is difficult to compare the allosteric effects of gallamine on [^{3}H]-QNB and [^{3}H]NMS in terms of order of potency, because the effects on [^{3}H]QNB are more complex. As we have reported previously for cardiac receptors [5], gallamine exerted a biphasic effect on the dissociation of [^{3}H]QNB from the m2 subtype (Figs. 3 and 4). The m1 receptor showed a qualitatively similar pattern at approximately 10-fold higher concentrations of gallamine (Fig. 4). The concentration of gallamine that *accelerated* [^{3}H]QNB dissociation to half-maximal extents were similar to those that caused half-maximal deceleration of [^{3}H]-NMS binding at m1 and m2 subtypes, respectively (compare Figs. 2 and 4). This *suggests* that these two effects may be mediated by a common site of interaction of gallamine. Using the same pharmacological argument, a distinct site may be responsible for the deceleration of the dissociation of [^{3}H]QNB. Binding of gallamine at this site either has no effect on [^{3}H]NMS dissociation or is hidden by a floor effect (dissociation is already slowed so much that further effects are not distinguishable). The apparent positive cooperativity in the binding profile of tetrahydroaminoacridine has led others to suggest that there may be more than one allosteric site per receptor [25].

The effects of gallamine on dissociation of both ligands were very sensitive to ionic strength. In the case of [^{3}H]QNB, higher ionic strength resulted in a sharp attenuation of the acceleratory component (Fig. 6). Nonetheless, the concentration-dependence of this effect continued to parallel the deceleratory effect on [^{3}H]NMS binding (compare Figs. 5 and 6). This leads us to believe that the second allosteric site is insensitive to ionic strength and powerfully decelerates the dissociation of [^{3}H]QNB, so that there is less *net* effect. As suggested above, the second site did not have an appreciable effect on [^{3}H]NMS dissociation, so that increasing ionic strength led to shifts toward higher gallamine concentrations, without changing the shapes of the curves (Fig. 5). The differences between the allosteric sensitivities of [^{3}H]NMS and [^{3}H]QNB make these ligands useful probes of receptor characteristics. For example, the set of curves in Fig. 6 provides insight into the lack of effect of 10^{-4} M gallamine on the dissociation of [^{3}H]QNB; the same concentration virtually stops the dissociation of [^{3}H]NMS. It is not that QNB is insensitive to gallamine, but rather that opposing effects are negating each other. The physiologically most interesting effects of allosteric modulation are ultimately those that affect agonist-mediated function, either by altering agonist binding or, equally likely, by altering the conformation of the agonist–receptor complex and, hence, its coupling efficiency. Investigators of such coupling phenomena should beware that different allosteric sites may be brought into play at 10^{-5} M gallamine and at 10^{-3} M gallamine, even though studies with [^{3}H]NMS suggest a continuum of effect. The postulation of a second allosteric site also provides insight into the allosteric effects of verapamil and *d*-tubocurarine. Verapamil was more than 10-fold less potent than *d*-tubocurarine at slowing the dissociation of [^{3}H]-NMS from the m2 site (Fig. 7, middle panel), but more than 10-fold *more* potent at slowing the dissociation of [^{3}H]QNB from the same site (Fig. 7, bottom panel). Thus, while verapamil exhibited about the same potency in these two assays, there was a 1000-fold discrepancy in the potencies of *d*-tubocurarine. We suggest that verapamil *only slows* the dissociation of [^{3}H]QNB, whereas *d*-tubocurarine follows a gallamine-like pattern (note that at the m2 site in Figs. 2 and 4 there was about a 2000-fold discrepancy between the concentrations of gallamine that *slowed* the dissociation of NMS and QNB). This seems to be a fundamental difference between the interactions of verapamil and those of gallamine and *d*-tubocurarine.

It has been suggested [26] that the allosteric modulation of brainstem (but not cortical) muscarinic receptors by gallamine may involve G protein(s). We have not observed effects of guanine nucleotides in our studies of allosteric effects [5]. Although the inclusion of Mg^{2+} in the assay did modulate the effects somewhat at the m2 subtype (Fig. 8), this is likely related to the effects of ionic strength (above). Treatment of transfected cells with pertussis toxin abolished the ability of guanine nucleotides to modulate agonist affinity but did not have any effect on allosteric modulation by gallamine. Whether pertussis toxin-insensitive G proteins may play a role in the allosteric regulation of the m2 or other subtypes remains to be determined. However, the present results are consistent with the idea that the allosteric site is part of the receptor protein *per se* [15, 24, 25]. It has been suggested that different ligands may bind to different sites within the same pocket of the receptor and that steric constraints may determine whether such binding is mutually exclusive (competitive) or not [24]. Steric hindrance might offer a simple explanation for the abilities of allosteric ligands to *slow* the kinetics of binding and dissociation of labeled ligands. However, the finding that gallamine and *d*-tubocurarine can accelerate the dissociation of [^{3}H]QNB (above) suggests that these drugs alter the conformation of the binding site.

In summary, we have shown that all of the known subtypes of muscarinic receptors are susceptible to allosteric regulation. The dramatic qualitative differences among the characteristics of verapamil, *d*-tubocurarine, and gallamine (at the m2 site) suggest that these agents must be able to elicit very different conformational changes in the receptor. Thus, it appears that there is the potential for a rich complexity of subtype-specific allosteric modulation of receptor function.

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