Biochemical Pharmacology, Vol. 53, pp. 795–800, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00814-3

Kinetic Studies of Co-Operativity at Atrial Muscarinic M_2 Receptors With an "Infinite Dilution" Procedure

Arthur Christopoulos, Alfred Lanzafame, Albrecht Ziegler and Fred Mitchelson†*

DEPARTMENT OF PHARMACEUTICAL BIOLOGY AND PHARMACOLOGY,
VICTORIAN COLLEGE OF PHARMACY (MONASH UNIVERSITY), PARKVILLE, 3052, VICTORIA, AUSTRALIA

ABSTRACT. The effects of two competitive antagonists and two allosteric ligands on the rate of dissociation of [^{3}H]N-methylscopolamine ([^{3}H]NMS) were studied at atrial muscarinic acetylcholine M_2 receptors by the technique of "infinite dilution." The dissociation rate for [^{3}H]NMS, initiated by diluting the incubation mixture in a 100-fold volume of buffer, was 0.61 ± 0.10 min^{-1}. Addition of the competitive antagonists, atropine or NMS, to the dilution medium did not alter the observed [^{3}H]NMS dissociation rate. In contrast, gallamine and the bisquaternary, heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl-ammonium bromide) (C_7/3′-phth), produced a concentration-dependent slowing of the dissociation rate of [^{3}H]NMS, with IC_{50} values of 7.5 μM and 196 nM, respectively. Gallamine exhibited an increased modulatory potency when equilibration with the tissue was allowed prior to dilution. The findings showed that the influence of low concentrations of allosteric modulators on the [^{3}H]NMS dissociation rate may be demonstrated separately from any effects on association rate, and that the contact time with the allosteric ligand may influence the extent of these effects. BIOCHEM PHARMACOL 53;6:795–800, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. muscarinic acetylcholine M_2 receptors; allosteric interaction; co-operativity; dissociation kinetics; gallamine; bisquaternary antagonist

Co-operativity in drug-receptor interactions is commonly demonstrated by observing a change in the dissociation rate of a radiolabelled competitive ligand on addition of an allosteric modulator in the presence of an excess of unlabelled competitive ligand [1]. The alternative technique of "infinite dilution" with buffer [2, 3] may also be used in place of excess competitive ligand to limit reassociation of the radioligand. This latter method offers an advantage, in that any influence of the allosteric modulator on the association of the excess unlabelled ligand is avoided.

In studies employing the former "excess ligand" kinetic procedure, it has recently been shown that low concentrations (*ca.* K_D) of some allosteric modulators resulted in apparent slowing of [^{3}H]NMS‡ dissociation from guinea-pig atrial muscarinic acetylcholine M_2 receptors [4]. This was only observed when the concentration of the excess competitive ligand used to limit [^{3}H]NMS reassociation was also low (*ca.* 30 × K_D). When higher concentrations (*ca.* 1000 × K_D) of the excess ligand were utilized, the modulatory effects of low concentrations of the allosteric ligands were no longer apparent. The difference was attributed to the limited ability of the allosteric modulators to retard the association of the unlabelled excess ligand, as well as the dissociation of the radioligand.

Because the "infinite dilution" technique eliminates the need for excess ligand, studies of dissociation kinetics with this method should provide a clearer picture of any effects of allosteric modulators on the dissociation of the radioligand alone. In the present study, the modulatory effects of gallamine and the bisquaternary C_7/3′-phth on the dissociation of [^{3}H]NMS from M_2 receptors in guinea-pig atrial homogenates were investigated by this technique.

MATERIALS AND METHODS

Radioligand Kinetic Procedure

Guinea pigs of either sex were killed by exsanguination and their hearts were rapidly removed and placed in ice-cold phosphate buffer (50 mM Na_2HPO_4, pH 7.4). Atria were then separated from ventricles, blotted dry, minced finely, weighed, mixed with 15 volumes of phosphate buffer and homogenized in an Ultra-Turrax set at 0.75 × maximum speed for 2 × 30 sec, with a 30-sec period of cooling between homogenization. Following centrifugation at −4°C for 10 min at 1000 × *g*, the supernatant was collected and used in the radioligand kinetic assays.

The radioligand dissociation kinetic protocol employed was as follows: A 3-mL aliquot of homogenate in 15 mL

* On sabbatical leave from the Department of Pharmacology, Christian-Albrechts University, Kiel, Germany.

† Corresponding author. Fred Mitchelson, Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy (Monash University, Parkville, 3052, Victoria, Australia.) Tel. (613)9903-9564; FAX (613)9903-9638.

‡ *Abbreviations:* NMS, N-methylscopolamine; C_7/3′-phth, heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl-ammonium bromide).

final volume of phosphate buffer, containing 0.3 nM [^{3}H]NMS, was equilibrated for 1 hr at 32°C. At this point, the preparation was diluted in a 100-fold volume of phosphate buffer, either in the absence or presence of a competitive antagonist or allosteric modulator at various concentrations, as specified in Results. Dissociation of the [^{3}H]NMS was then followed by taking 50-mL aliquots, in duplicate, of the continuously-stirred mixture at various time intervals. After filtering, radioactivity was determined by scintillation counting (5 mL Packard Filter-Count/filter).

Two additional variations of this protocol were employed when studying the effects of gallamine. In one protocol, gallamine was allowed to equilibrate together with the [^{3}H]NMS for 3 hr before dilution was undertaken. The diluent contained sufficient allosteric modulator to maintain the same concentration throughout the experiment. The other protocol was as above, except that the homogenate was allowed to equilibrate with the [^{3}H]NMS for 1 hr before the addition of the allosteric modulator for a further 3 hr.

Equilibrium Binding Procedure

To determine the equilibrium binding properties of gallamine, under the conditions employed in the kinetic study, some inhibition binding experiments were also conducted.

Atria were prepared as described above. The experiments were conducted in phosphate buffer (as above) and involved incubating 0.2-mL aliquots of homogenate with increasing concentrations of gallamine (0.01–100 μM), in the presence of a fixed concentration of [^{3}H]NMS. Each experiment utilized a two-curve assay, where the fixed concentration of radioligand was 0.3 nM (*ca.* K_D) for the first inhibition curve and 3 nM (*ca.* $10 \times K_D$) for the second inhibition curve. Incubation (1 mL final volume/tube) was then allowed to proceed for 3 hr at 32°C before termination, by vacuum filtration. Nonspecific binding was defined using atropine (10 μM). Radioactivity was determined as described above.

Data Evaluation

Radioligand dissociation rates were evaluated by nonlinear regression analysis of the exponential decay using the computer program PRISM 2.0 (GraphPad Software, San Diego, CA) with the following equation:

$$Y = (a - b) \cdot e^{-k_{off}X} + b$$

where a represents the vertical span of the data, b is the plateau value, X is the time (min), and k_{off} is the dissociation rate constant (min^{-1}). Data are given as the mean ± standard error of the mean (mean ± SEM). Statistical significance was determined using an unpaired t-test (2-tailed), with values of $P < 0.05$ being considered significant. Data were also analyzed according to an extended version of the above equation that allowed for biexponential dissociation. In all cases, however, the results were better described by the monoexponential model.

The resulting mean k_{off} values for [^{3}H]NMS in the presence of various concentrations of the allosteric modulators were converted to percentages of the control k_{off} of the radioligand and plotted against the corresponding concentration of modulator. The concentration of modulator resulting in a half-maximal reduction of radioligand dissociation rate (IC_{50}) was obtained by fitting the following four-parameter logistic function through the points *via* nonlinear regression using PRISM:

$$y = a + \frac{b}{1 + 10^{-n(\log IC_{50} - \log [X])}}$$

where a is the minimum of the curve, b is the vertical span, n is the mid-point slope, and [X] is the concentration of allosteric modulator.

For the equilibrium binding data, the program SCIENTIST 2.01 (MicroMath Software, Salt Lake City, UT) was used to simultaneously analyse both inhibition curves, obtained from each experiment, based on the following equation [5]:

$$\frac{Y}{Y_0} = \frac{[A]}{[A] + K_A\left(\frac{K_Z + [Z]}{K_Z + [Z]/\alpha'}\right)}$$

where Y/Y_0 represents fractional receptor occupancy, [A] and [Z] represent the concentrations, K_A and K_Z represent the equilibrium dissociation constants of the radioligand and allosteric modulator, respectively, and α' represents the heterotropic co-operativity factor between the two inhibitors. The values of [A], [Z] and K_A were entered, and K_Z was estimated as logarithms.

Drugs

The following drugs were used: (–)-[N-methyl-^{3}H]scopolamine methyl chloride (Amersham, Amersham, UK), atropine sulphate, (–)-N-methyl scopolamine bromide, gallamine triethiodide (Sigma Chemical Company, St. Louis, MO) and heptane-1,7,bis-(dimethyl-3′-phthalimidopropyl-ammonium bromide), C_7/3′-phth (Institute of Drug Technology, Boronia, Victoria, Australia).

RESULTS

Effects of Competitive Antagonists

Addition of the competitive muscarinic receptor antagonists, atropine (1 or 100 nM) or NMS (0.3 or 30 nM), to the dilution buffer did not produce a significant ($P > 0.05$) alteration of the [^{3}H]NMS dissociation rate, as shown in Table 1.

TABLE 1. Dissociation rate constant (k_{off}) parameters for [^{3}H]NMS in the absence or presence of various concentrations of competitive antagonists in guinea-pig atrial homogenates

Competitive antagonist	Concentration (nM)	k_{off} (min^{-1})*	n†
–	–	0.61 ± 0.10	6
Atropine	1	0.50 ± 0.12	3
	100	0.64 ± 0.04	3
NMS	0.3	0.52 ± 0.03	5
	30	0.60 ± 0.07	3

* Dissociation rate constant of [^{3}H]NMS as determined by computer analysis (see Data Evaluation); † number of experiments.

Effect of C_7/3′-phth

The bisquaternary C_7/3′-phth (0.3–100 μM) produced a concentration-dependent slowing of the [^{3}H]NMS dissociation (Fig. 1). The resulting k_{off} values for [^{3}H]NMS are shown in Table 2 and are also expressed as a percentage of the control k_{off} value of [^{3}H]NMS. In the presence of the lowest concentration of C_7/3′-phth employed, 0.1 μM (*ca.* K_D), the observed k_{off} of [^{3}H]NMS was not significantly different from control ($P > 0.05$). The IC_{50} value of C_7/3′-phth for reducing the k_{off} of [^{3}H]NMS was found to be 196 nM (Fig. 2). The midpoint slope value of 1.35 ± 0.23 was not significantly different from unity ($P > 0.05$).

Effect of Gallamine

Gallamine (10–100 μM) also slowed the dissociation of the [^{3}H]NMS in a concentration-dependent manner (Table 3).

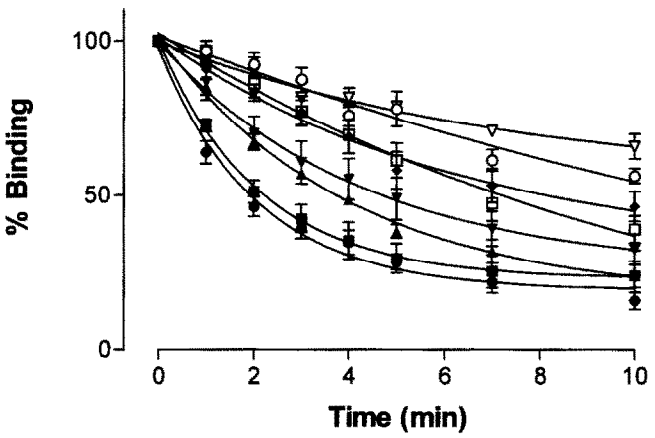

FIG. 1. Effect of C_7/3′-phth on the dissociation rate of [^{3}H]NMS in guinea-pig atria. Homogenates were equilibrated with 0.3 nM [^{3}H]NMS in 50 mM phosphate buffer, pH 7.4, at 32°C for 1 hr (control dpm: 2290.6 ± 396) before dissociation was initiated *via* dilution in 100-volumes of phosphate buffer and followed from time 0 min in the absence (●) or presence of the following concentrations of C_7/3′-phth: 0.1 μM (■), 0.3 μM (▲), 1 μM(▼), 3 μM (♦), 10 μM (□), 30 μM (○), 100 μM (▽). Each point represents the mean of 3–6 experiments performed in duplicate. The regressions are plotted using the calculated mean k_{off} values.

TABLE 2. Dissociation rate constant (k_{off}) parameters for [^{3}H]NMS in the absence or presence of various concentrations of C_7/3′-phth in guinea-pig atrial homogenates

C_7/3′-phth (μM)	k_{off} (min^{-1})*	% Control k_{off}	n†
–	0.61 ± 0.10	100	6
0.1	0.48 ± 0.04	79	4
0.3	0.24 ± 0.06‡	39	4
1	0.15 ± 0.03‡	25	3
3	0.09 ± 0.02‡	15	4
10	0.07 ± 0.02‡	12	4
30	0.06 ± 0.03‡	10	4
100	0.04 ± 0.02‡	7	4

* Dissociation rate constant of [^{3}H]NMS as determined by computer analysis (see Data Evaluation).
† Number of experiments.
‡ Significantly different ($P < 0.05$) from control k_{off}.

Lower concentrations of gallamine (1 or 3 μM) did not cause a significant reduction ($P > 0.05$) of the k_{off} of the radioligand (Table 3). The IC_{50} value for the effect of gallamine on radioligand dissociation was 7.5 μM (Fig. 2). The value of the midpoint slope was 1.19 ± 0.06 and was not significantly different from unity ($P > 0.05$).

The lack of observed effects with the lower concentrations of gallamine may have been due to inadequate equilibration periods. This was examined by allowing gallamine (0.1 or 0.3 μM) and [^{3}H]NMS to equilibrate for 3 hr with or without a 1-hr pre-equilibration of [^{3}H]NMS alone. The results are presented in Table 3, where it can be seen that, with either experimental protocol, gallamine (0.3 μM) was able to significantly ($P < 0.05$) slow the dissociation of [^{3}H]NMS. Experiments examining [^{3}H]NMS dissociation rate after a 4-hr equilibration period did not show any significant ($P > 0.05$) change in the control k_{off} value when compared to that obtained after a 1-hr equilibration period (data not shown).

Some studies were also conducted to examine the effect of gallamine on the equilibrium binding of [^{3}H]NMS. Gallamine exhibited a limited ability to inhibit the binding of the radioligand, especially when the concentration of the latter was *ca.* 10 × K_D (Fig. 3). Analysis of the data according to an allosteric model (see Data Evaluation) yielded a log dissociation constant for gallamine (log K_Z) with the unoccupied receptor of −6.86 ± 0.05 ($n = 3$) and a heterotropic co-operativity factor (α′) of 14.86 ± 1.94. This corresponded to an apparent dissociation constant (α′ × K_Z) for gallamine with the [^{3}H]NMS-occupied receptor of 1.96 μM.

DISCUSSION

The dissociation rate constant of 0.61 min^{-1} for [^{3}H]NMS obtained by the "infinite dilution" method from this study was within the range of values (0.57–0.72 min^{-1}) obtained

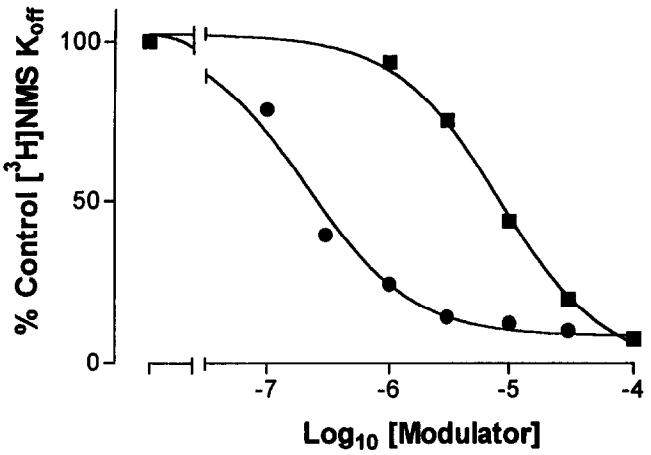

FIG. 2. Plot of the reduction in the k_{off} of [^{3}H]NMS produced by various concentrations of C_7/3′-phth (●) or gallamine (■) at guinea-pig atrial M_2 receptors.

previously [4] by the alternative "excess ligand" ligand method. A statistical comparison of multiple group means [6] showed no significant difference ($P > 0.05$) between the values obtained. Thus, a 100-fold dilution of the homogenate in buffer gave a comparable dissociation rate for [^{3}H]NMS to that observed in the presence of a 1000 × K_D excess of competitive ligand (NMS or atropine) [4], suggesting that maximal radioligand dissociation was achieved. In the present experiments, it was also observed that addition of a competitive antagonist, such as NMS or atropine, to the dilution medium did not significantly alter the dissociation rate of the [^{3}H]NMS. This provides further support for considering that maximal radioligand dissociation had been achieved by dilution and, furthermore, illustrates the fact that a competitive antagonist will not affect the dissociation rate of the radioligand any further under the dilution conditions.

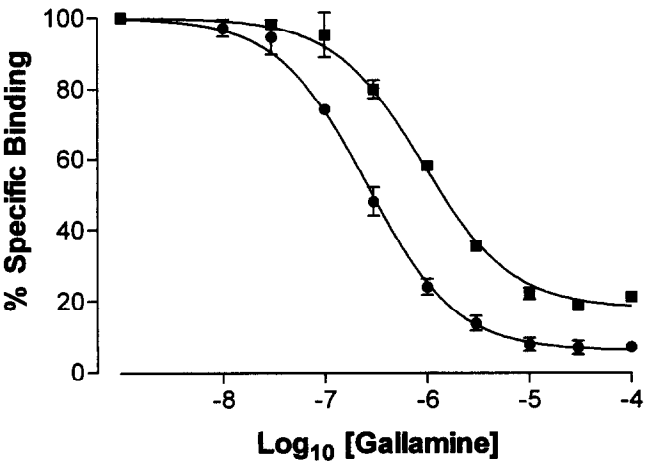

FIG. 3. Effect of gallamine on the equilibrium binding of [^{3}H]NMS, 0.3 nM (●, control dpm: 1994 ± 472) and 3 nM (■; control dpm: 4190 ± 658), in guinea-pig atria. Homogenates were incubated in 50 mM phosphate buffer, pH 7.4, at 32°C for 3 hr. Normalized curves represent simultaneous fits for an allosteric modulator (log K_z = −6.86 ± 0.05; n = 3) interacting with a competitive antagonist (log K_A = −9.52); the co-operativity factor (α′) was estimated as 14.86 ± 1.94 (see Data Evaluation).

TABLE 3. Dissociation rate constant (k_{off}) parameters for [^{3}H]NMS in the absence or presence of various concentrations of gallamine in guinea-pig atrial homogenates

Gallamine (μM)	k_{off} (min^{-1})*	% Control k_{off}	n†
–	0.61 ± 0.10	100	6
1	0.57 ± 0.02	93	3
3	0.46 ± 0.05	76	3
10	0.27 ± 0.04¶	44	4
30	0.12 ± 0.02¶	20	5
100	0.04 ± 0.01¶	7	5
0.3§	0.33 ± 0.05¶	54	4
0.1‖	0.57‡	93	2
0.3‖	0.30 ± 0.09¶	49	4

* Dissociation rate constant of [^{3}H]NMS as determined by computer analysis (see Data Evaluation).

† Number of experiments.

‡ Mean of values 0.48 and 0.65 min^{-1}.

§ Pre-equilibration with gallamine as well as [^{3}H]NMS for 3 hr before dilution (see Methods).

‖ Pre-equilibration with gallamine for 3 hr as well as [^{3}H]NMS for 4 hr before dilution (see Methods).

¶ Significantly different ($P < 0.05$) from control k_{off}.

On the other hand, an allosteric modulator, by binding to an accessory site, may be expected to induce a conformational change in the receptor that could be manifested as either an increase or a decrease in radioligand dissociation [3]. For example, it has been shown that the presence of unlabelled insulin further increased the dissociation rate of radiolabelled insulin from its binding site following dilution, indicative of negative, homotropic co-operativity [7]. In contrast, C_7/3′-phth and gallamine displayed a concentration-dependent retardation of the dissociation of [^{3}H]NMS in the present experiments. This is in agreement with previous kinetic studies using these modulators, based on the excess ligand method [1, 4, 8–11]. Calculation of the IC_{50} values for the two ligands, from the data in the present study, showed C_7/3′-phth to be the more potent allosteric modulator of [^{3}H]NMS dissociation.

Allosteric interactions at muscarinic receptors are considered to involve the formation of a ternary complex between the allosteric and orthosteric ligands and the receptor monomer [5, 8]. Accordingly, gallamine and C_7/3′-phth have been shown to act as negative heterotropic modulators of the equilibrium binding of both agonists and competitive antagonists at the orthosteric site [8, 11–14], and this was confirmed, for gallamine, in the present study. The finding that gallamine or C_7/3′-phth slowed the dissociation rate of [^{3}H]NMS implies that they must exhibit even more pronounced effects on ligand association, if they act as negative allosteric modulators. Some indication that this

does occur was obtained in a previous study employing excess, orthosteric (i.e. competitive) ligand to promote dissociation [4]. The dissociation rate of [^{3}H]NMS observed in the presence of a 30-fold excess of orthosteric ligand was reduced to a significantly greater extent by either allosteric ligand than that observed in the presence of a 1000-fold excess of orthosteric drug. This difference was attributed to an additional effect of the allosteric ligands on the association rate of the excess ligand, being more evident when lower concentrations of excess ligand were employed. This phenomenon was not a factor in the present experiments, as dissociation was induced by dilution of the incubation medium.

Because gallamine has been shown to require a long equilibration with [^{3}H]NMS at muscarinic receptors [8], some experiments were conducted in which both ligands were allowed to equilibrate with the receptor for 3 hr. Recently, Tuček and Proška [15] have discussed the evidence for, and importance of, an obligatory binding sequence for some allosteric modulators, such as alcuronium, when combined with competitive ligands at muscarinic receptors. They suggested that the modulator may interfere sterically with the traditional orthosteric site recognised by competitive ligands, as well as exerting co-operative, allosteric effects. If this phenomenon was present with gallamine, the concomitant occurrence of these two distinct events may obscure the co-operative modulatory effects of the ligand on [^{3}H]NMS dissociation kinetics. Hence, experiments were also conducted where [^{3}H]NMS was allowed to equilibrate with the homogenate for 1 hr before the addition of gallamine for a further 3 hr, followed by dilution. In either type of modified protocol, gallamine, at a concentration of 0.3 μM, now appeared effective in slowing the dissociation of [^{3}H]NMS, whereas, in the initial experiments discussed above, 3- to 10-fold higher concentrations of gallamine did not exert a significant effect. These findings suggest that the IC_{50} value of gallamine for slowing dissociation is overestimated if allowances are not made for equilibration between the interacting ligands and their respective binding sites.

Lazareno and Birdsall [16] have described theoretical conditions under which the concentration of allosteric modulator producing 50% inhibition of radioligand dissociation may be used to derive the co-operativity factor (α'). In the present study, the dissociation curves were monophasic, suggesting that such an analysis could be employed. The IC_{50} value of 7.5 μM for the modulatory potency of gallamine on the dissociation of [^{3}H]NMS appeared to decrease to *ca.* 0.3 μM on prolonged incubation (see Table 3), indicative of slow association kinetics. Furthermore, neither value agreed with the apparent dissociation constant of 1.96 μM ($\alpha' \times K_Z$) estimated from the equilibrium-binding studies. The findings are difficult to interpret without knowledge of the kinetic constants for the allosteric modulator itself [16]. Thus, quantitative estimates of co-operativity from studies such as those depicted in Fig. 2 do not take into account equilibration of the allosteric ligand with the receptor or its effects on ligand association. A combination of both "excess ligand" and "infinite dilution" experiments performed under appropriate equilibrium conditions should provide a more exhaustive means of assessing the effects of allosteric modulators on dissociation and association rates of competitive ligands at muscarinic receptors.

In conclusion, use of the "infinite dilution" procedure has demonstrated that both C_7/3′-phth and gallamine slow the dissociation of [^{3}H]NMS from atrial M_2 receptors at low concentrations. However, the contact time of the allosteric ligand must also be considered when determining its effect on competitive radioligand dissociation.

This work was funded by a grant from the National Health and Medical Research Council of Australia.

References

1. Lee NH and El-Fakahany EE, Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem Pharmacol* **42:** 199–205, 1991.
2. Delean A and Rodbard D, Kinetics of cooperative binding. In: *The Receptors: A Comprehensive Treatise* (Ed. O'Brien, RD), pp. 143–192. Plenum Press, New York, 1979.
3. Henis YI, Kloog Y and Sokolovsky M, Allosteric interactions of muscarinic receptors and their regulation by other membrane proteins. In: *The Muscarinic Receptors* (Ed. Brown, JH), pp. 377–418. Humana Press, Inc., New York, 1989.
4. Christopoulos A and Mitchelson F, [^{3}H]N-methylscopolamine dissociation from muscarinic receptors affected by low concentrations of allosteric modulators. *Eur J Pharmacol (Molec Pharmacol Sect)* **290:** 259–262, 1995.
5. Ehlert FJ, Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33:** 187–194, 1988.
6. Miller RG, *Simultaneous Statistical Inference*. McGraw Hill, New York, 1966.
7. DeMeyts P, The structural basis of insulin and insulin-like growth factor-I (IGF-I) receptor binding and negative cooperativity, and its relevance to mitogenic versus metabolic signalling, *Diabetologia* **37:** S135–S148, 1994.
8. Stockton JM, Birdsall NJM, Burgen ASV and Hulme EC, Modification of the binding properties of muscarinic receptors by gallamine. *Mol Pharmacol* **23:** 551–557, 1983.
9. Waelbroeck M, Robberecht P, De Neff P and Christophe J, Effects of *d*-tubocurarine on rat cardiac muscarinic receptors. A comparison with gallamine. *J Recept Res* **8:** 787–808, 1988.
10. Michel AD, Delmondo RE, Lopez M and Whiting RL, On the interaction of gallamine with muscarinic receptor subtypes. *Eur J Pharmacol* **182:** 335–345, 1990.
11. Christopoulos A and Mitchelson F, Assessment of the allosteric interaction of the bisquaternary heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl ammonium bromide), at M_1 and M_2 muscarine receptors. *Mol Pharmacol* **46:** 105–114, 1994.
12. Lüllmann H, Ohnesorge FK, Schauwecker G-C and Wassermann O, Inhibition of the actions of carbachol and DFP on

guinea pig isolated atria by alkane bis-ammonium compounds. *Eur J Pharmacol* **6:** 241–247, 1969.
13. Clark AL and Mitchelson F, The inhibitory effects of gallamine on muscarinic receptors. *Br J Pharmacol* **58:** 323–331, 1976.
14. Choo LK and Mitchelson F, Characterization of the antimuscarinic effect of heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl ammonium bromide). *Eur J Pharmacol* **162:** 429–435, 1989.
15. Tuček S and Proška J, Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol Sci* **16:** 205–212, 1995.
16. Lazareno S and Birdsall NJM, Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48:** 362–378, 1995.