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Using a Radioalloster to Test Predictions of the Cooperativity Model for Gallamine Binding to the Allosteric Site of Muscarinic Acetylcholine M_2 Receptors

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ABSTRACT

The muscarinic M_2 receptor contains an orthosteric and an allosteric site. Binding of an allosteric agent may induce a shift α of the equilibrium dissociation constant K_D of a radioligand for the orthosteric site. According to the cooperativity model, the K_A of alloster binding is expected to be shifted to an identical extent depending on whether the orthosteric site is occupied by the orthoster or not. Here, the novel radioalloster [^{3}H]dimethyl-W84 (*N*,*N*′-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2*H*-isoindol-2-yl)propyl]-*N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexanediaminium diiodide) was applied to directly measure the K_A shift induced for the prototype allosteric modulator gallamine by binding of *N*-methylscopolamine (NMS) to the orthosteric site of porcine heart M_2 receptors (4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4; 23°C; data are means ± S.E.). First, in the common way, the concentration-dependent inhibition by gallamine of [^{3}H]NMS equilibrium binding was measured and analyzed using the cooperativity model, which yielded for the affinity of gallamine binding at free receptors a pK_A= 8.35 ± 0.09 and a cooperativity factor α = 46 (n = 5). The dissociation constant for gallamine binding at NMS-occupied receptors was predicted as $p(\alpha \cdot K_A)$ = 6.69. Labeling of the allosteric site by [^{3}H]dimethyl-W84 allowed the measure of competitive displacement curves for gallamine. The K_i for gallamine at free receptors amounted to $pK_{i,-NMS}$ = 8.27 ± 0.39 (n = 5), which is in line with the prediction of the cooperativtiy model. In the presence of 1 μM NMS, to occupy the orthosteric site, gallamine displaced [^{3}H]dimethyl-W84 with $pK_{i,+NMS}$ = 6.60 ± 0.19 (n = 3). Thus, the NMS-induced pK_i shift amounted to 47, which matches the predicted value of α = 46. These results validate the cooperativity model.

Allosteric modulation of ligand binding in muscarinic acetylcholine receptors has been intensively studied (cf. Lee and El-Fakahany, 1991; Tuček and Proška, 1995; Ellis, 1997; Christopoulos et al., 1998; Holzgrabe and Mohr, 1998 for review). Other G protein-coupled receptors found sensitive for allosteric modulation include adenosine A_1 receptors (Bruns and Fergus, 1990), α_2 adrenoceptors (Nunnari et al., 1987; Wilson et al., 1992; Leppik et al., 1998), and dopamine D_2 receptors (Hoare and Strange, 1996). Allosteric modulation of G protein-coupled receptors offers the possibility to increase the action of orthosteric agonists (e.g., Bruns and Fergus, 1990; Doležal and Tuček, 1998) with an extent of subtype selectivity not achieved before (Birdsall et al., 1997, 1999).

The muscarinic acetylcholine M_2 receptor is very sensitive to allosteric modulation (e.g., Lee and El-Fakahany, 1991; Christopoulos et al., 1998; Holzgrabe and Mohr, 1998 for review) and may be considered as a model system to study allosteric interactions at G protein-coupled receptors.

Until very recently, the receptor binding characteristics of an allosteric agent could not be measured directly but were deduced from the effects induced by the allosteric compound on the binding of an orthosteric radioligand. The effect on the equilibrium binding of the radioorthoster was analyzed by means of the ternary complex model of allosteric interactions, the so-called "cooperativity model", which has been introduced into the muscarinic field by Stockton et al. (1983), simplified by Ehlert (1988), and further developed by Lazareno and Birdsall (1995) to cover an array of different experimental situations. The alloster-induced K_D shift of the orthosteric ligand is indicated by the cooperativity factor α. The cooperativity model predicts that α is reciprocal in nature, i.e., α equals the shift in induced by the orthosteric ligand. However, to date, this prediction has not been tested by direct binding measurements at the allosteric site. Recently, we found a radioligand for labeling the allosteric site in muscarinic acetylcholine M_2 receptors, i.e., [^{3}H]dimethyl-

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ABBREVIATIONS: M_2 receptor, M_2 subtype of muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; dimethyl-W84, *N*, *N*′-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2*H*-isoindol-2-yl)propyl]-*N*, *N*,*N*′,*N*′-tetramethyl-1,6-hexanediaminium diiodide; K_A, equilibrium dissociation constant for alloster binding to free receptors.

W84 (*N*,*N*′-bis[3-(1,3-dihydro-1,3-dioxo-4-methyl-2H-isoindol-2-yl)propyl]-*N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexanediaminium diiodide) (Tränkle et al., 1998). Applying this compound, we aimed to find out whether the orthoster-induced shift of alloster binding affinity occurs as predicted by the cooperativity model. Therefore, we studied the interaction of the orthosteric ligand *N*-methylscopolamine (NMS) with the prototype allosteric modulator gallamine. We chose gallamine because it acts via the "common site" (Ellis and Seidenberg, 1992; Tränkle and Mohr, 1997) and because gallamine is known to display an intermediate negative cooperativity with NMS that should be large enough to be measurable in [^{3}H]dimethyl-W84 binding experiments. First, we determined the effect of gallamine on the binding of the orthosteric ligand [^{3}H]NMS in the "conventional" way and obtained the cooperativity factor α by an analysis of the data according to the cooperativity model. Second, applying the radioalloster [^{3}H]dimethyl-W84, we measured in competition experiments the K_i of gallamine binding at the allosteric site either in the absence or in the presence of NMS (at a concentration saturating the orthosteric site). According to the cooperativity model, the orthoster-induced shift of the K_i of alloster binding should match the cooperativity factor α.

Materials and Methods

Membrane Preparation. Homogenates of porcine myocardium were prepared as described previously in detail (Tränkle et al., 1996). In short, at an ambient temperature of 3–6°C, pieces of the ventricular myocardium of freshly excised hearts of domestic pigs were washed in sucrose solution (0.32 M) and homogenized using a Waring Blender and a Potter-Elvejhem homogenizer. After centrifugation of the homogenate for 11 min at 300*g* (2000 rpm in a Beckman rotor 35; Beckman-Coulter, Fullerton, CA), the resulting supernatants were pelleted for 15 min at 20,900*g* (13,000 rpm in a Beckman rotor 35). The final pellets were resuspended in a buffer composed of 4 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4 (Na, K, P_i-buffer). Aliquots of 1 ml in volume were shock-frozen in liquid nitrogen and stored at −80°C. The protein content ranged between 4.5 and 9 mg/ml membrane suspension.

Binding Assays. Binding experiments were carried out applying a centrifugation assay as described previously for measurement of [^{3}H]dimethyl-W84 binding (Tränkle et al., 1998). Homogenate at a protein concentration of from 300 to 700 μg/ml was incubated in a volume of 1.5 ml with [^{3}H]dimethyl-W84 or [^{3}H]NMS, respectively, in the Na, K, P_i-buffer at a temperature of 23°C. The incubation time was 2 h unless otherwise indicated. [^{3}H]dimethyl-W84 experiments were carried out either in the absence or in the presence of 1 μM NMS to occupy the orthosteric binding site of the M_2 receptor and 1 μM physostigmine was applied to exclude an interaction of [^{3}H]dimethyl-W84 with acetylcholinesterase. At the indicated concentrations, neither NMS nor physostigmine interacted with the allosteric site of the [^{3}H]NMS-occupied M_2 receptor (data not shown). Membranes were separated by centrifugation with 20,900*g* (15,300 rpm) for 20 min at 23°C (Beckman rotor model F241.5). After quickly rinsing the pellet with 1.5 ml of cold Na, K, Pi-buffer to remove residual radioactivity from the tube wall, the pellet was resuspended in 1.5 ml of buffer and transferred into a scintillation vial filled with 10 ml of Ready Protein (Beckman) for liquid scintillation counting.

Effects of Gallamine on [^{3}H]Dimethyl-W84 Binding. [^{3}H]dimethyl-W84 was applied at a concentration of 0.3 nM. In the experiments with 1 μM NMS, the orthosteric ligand was allowed to equilibrate for 5 to 10 min with the receptors before [^{3}H]dimethyl-W84 was added to the assay. As shown previously (Tränkle et al., 1998), [^{3}H]dimethyl-W84 binding consists of three fractions. Nonspecific, nonsaturable [^{3}H]dimethyl-W84 binding as determined in the presence of high concentrations of unlabeled allosters amounts to about 20% of total [^{3}H]dimethyl-W84 binding (Tränkle et al., 1998). In the following, it is focused on the saturable binding, which is composed of a specific and a nonspecific fraction. The binding constant of specific [^{3}H]dimethyl-W84 binding (about 20–30% of the total) as determined in homologous competition experiments, amounted in the absence of NMS to (mean ± S.E.) $pK_D = 8.89 \pm 0.18$ (four experiments), and in the presence of 1 μM NMS, to $pK_D = 8.74 \pm 0.08$ (seven experiments) respectively (Tränkle et al., 1998). The effect of gallamine on the binding of [^{3}H]dimethyl-W84 (0.3 nM) was determined under identical conditions in heterologous competition experiments.

Effects of Gallamine on [^{3}H]NMS Binding. Binding of [^{3}H]NMS (0.2 nM) under control conditions was investigated by homologous competition experiments. Nonspecific [^{3}H]NMS binding was determined in the presence of 1 μM atropine and did not exceed 10% of total binding. The −log equilibrium dissociation constant pK_D amounted to 9.68 ± 0.10 (mean ± S.E.; eight experiments). The effect of gallamine on the equilibrium binding of the orthosteric radioligand was determined in inhibition experiments carried out simultaneously at 0.18 and 1.55 nM [^{3}H]NMS. The incubation time sufficient to obtain equilibrium binding of [^{3}H]NMS in the presence of gallamine was deduced from the action of gallamine on [^{3}H]NMS dissociation according to an equation adopted from Lazareno and Birdsall (1995):

$$t_{0.5obsA} = t_{0.5off} \cdot \left(1 + \frac{1}{EC_{50,diss}} \cdot A\right)$$

$t_{0.5obsA}$ is an estimate of radioorthoster association half-life time in the presence of allosteric modulator A, $t_{0.5off}$ is the half-life time of radioorthoster dissociation in the absence of allosteric modulator, and $EC_{50,diss}$ indicates the modulator concentration at which the half-life time of radioorthoster dissociation is doubled. At the highest gallamine concentration of 3 μM, a 6-h incubation period represented six times $t_{0.5obsA}$ and was considered sufficient to reach equilibrium.

Data Analysis. Data from experiments not involving the application of the ternary complex model of allosteric interactions were analyzed individually by computer-aided, nonlinear regression analysis using Prism ver. 3.0 (GraphPad, San Diego, CA). Analysis of homologous and heterologous competition data obtained with the respective radioligand was based on the general Hill equation. Because the observed Hill coefficients did not differ significantly from unity (partial *F* test; $P > .05$; data not shown), IC_{50} values were determined from curve fits with n_H fixed to 1. The binding parameter K_D was calculated according to DeBlasi et al. (1989). K_i values for the inhibitory action of gallamine on [^{3}H]dimethyl-W84 binding were obtained from IC_{50} values according to Cheng and Prusoff (1973).

Analysis of the effect of gallamine on the binding of [^{3}H]NMS was based on the ternary complex model of allosteric interactions according to Ehlert (1988) with the equation:

$$B = B_0 \cdot \frac{(L + K_D)}{\left\{L + K_D \cdot \frac{(K_A + A)}{(K_A + A/\alpha)}\right\}}$$

where B_0 denotes the equilibrium binding of a fixed radioligand concentration in the absence of allosteric ligand A, K_D is the equilibrium dissociation constant of the orthosteric ligand at the free receptor, K_A is the equilibrium dissociation constant of the allosteric ligand at the free receptor, and α is the cooperativity factor for the interaction between the allosteric modulator and the radioligand.

Simultaneous nonlinear fitting of inhibition curves obtained at the low and the high concentration of [^{3}H]NMS with, as two independent variables, the concentrations of gallamine and [^{3}H]NMS, respectively, was performed applying Sigma Plot for Windows (version 4.00; SPSS, Erkrath, Germany).

Drugs. Synthesis of dimethyl-W84 has been described previously (Tränkle et al., 1998). The synthesis of the radiolabeled compound [^{3}H]dimethyl-W84 was carried out by Amersham Life Science (Braunschweig, Germany). The radiochemical purity was 97%, and the specific activity amounted to 168 Ci/mmol = 6.22 Tbq/mmol. [^{3}H]NMS was purchased from DuPont/NEN (Bad Homburg, Germany), and atropine sulfate, (−)scopolamine *N*-methylbromide, and gallamine triethiodide were obtained from Sigma Chemicals (Munich, Germany).

Results and Discussion

Figure 1 depicts the effect of increasing concentrations of the allosteric modulator gallamine on the binding of the orthosteric ligand [^{3}H]NMS at a low (0.18 nM) and a high (1.55 nM) concentration. At both [^{3}H]NMS concentrations, gallamine reduced radioligand binding concentration-dependently, indicating its negative cooperativity with NMS. Whereas at the lower radioligand concentration, the gallamine inhibition curve approached the zero level of [^{3}H]NMS binding, inhibition by gallamine at the higher concentration of [^{3}H]NMS was incomplete and plateaued above the zero level, thus revealing the allosteric character of the interaction. The ternary complex model of allosteric interactions according to Ehlert (1988) was applied for simultaneous curve fitting to the two sets of data. The −log value of the equilibrium dissociation constant for gallamine binding to free receptors was computed to amount to $pK_A = 8.35 \pm 0.09$ (mean ± S.E.; five experiments), and the factor of cooperativity between gallamine and [^{3}H]NMS was $\alpha = 46$. This α value means that gallamine binding to the receptor is accompanied by a 46-fold decrease in [^{3}H]NMS binding affinity compared with [^{3}H]NMS binding to free receptors. Because cooperativity is reciprocal in nature, this finding predicts that the affinity constant of gallamine at NMS occupied receptors is decreased by the same factor: $p(\alpha \cdot K_A) = 6.69$.

To check the predictions, we applied the radioalloster [^{3}H]dimethyl-W84 (0.3 nM) to measure the K_i of gallamine at free and NMS-liganded M_2 receptors (Fig. 2). Because there is a negative cooperativity between gallamine and NMS (cf. Fig. 1), the question arises whether high concentrations of gallamine reduce occupation of the orthosteric site by 1 μM NMS to a relevant extent. In the absence of gallamine, 1 μM NMS ($pK_D = 9.68$) leads to a fractional receptor occupancy of 0.9998. From the characteristics of the inhibition of [^{3}H]NMS equilibrium binding by gallamine (see Fig. 1), it can be derived by using the cooperativity model that the fractional receptor occupancy by 1 μM NMS in the presence of the highest applied concentration of gallamine (1 mM) amounts to 0.9905. Accordingly, occupancy of the orthosteric site by NMS is maintained even at high concentrations of gallamine. In other words, the NMS receptor complex can be considered as an entity that represents the binding site of [^{3}H]dimethyl-W84 and gallamine.

As reported recently (Tränkle et al., 1998), inhibition of [^{3}H]dimethyl-W84 binding by increasing concentrations of gallamine at NMS-occupied M_2 receptors proceeds in a biphasic fashion (Fig. 2, inset), with the high-affinity component representing specific binding to the allosteric site. The rather high nonspecific binding is probably due to the use of centrifugation for separating the membranes instead of filtration with subsequent washes. However, the kinetics of [^{3}H]dimethyl-W84 binding at NMS-occupied receptors are probably very fast (Tränkle and Mohr, 1997) and, therefore, the centrifugation procedure is currently the only feasible experimental approach to measure radioalloster binding at NMS-occupied M_2 receptors under equilibrium conditions (Hulme, 1992). In the absence of NMS, the biphasic shape of the curve for inhibition of [^{3}H]dimethyl-W84 binding by gallamine was maintained. Under both conditions, with and without NMS, there was a plateau between the two components of saturable [^{3}H]dimethyl-W84 binding at a gallamine concentration of 3 μM. By defining nonspecific [^{3}H]dimethyl-W84 binding as the binding in the presence of 3 μM gallamine, the results depicted in the main part of Fig. 2 focus on the high-affinity specific component of [^{3}H]dimethyl-W84 binding. The curves have the shape of competitive displacement curves. It is obvious from Fig. 2 that 1 μM NMS occupying the orthosteric receptor site induced a pronounced

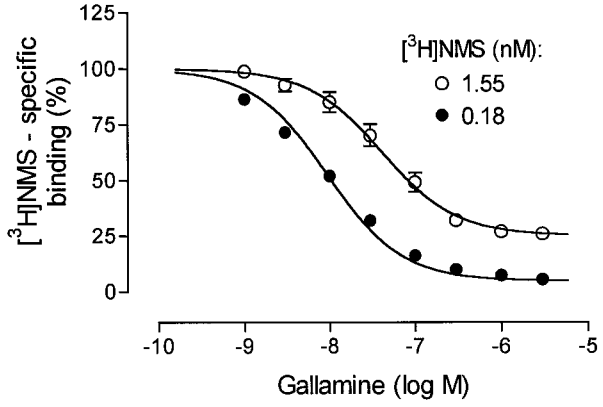

Fig. 1. Effect of gallamine on the specific binding of [^{3}H]NMS at 0.18 nM and 1.55 nM to M_2 acetylcholine receptors in porcine myocardial membranes. Data are expressed in percentage of [^{3}H]NMS binding in the absence of gallamine (fractional receptor occupancy 0.46 and 0.88, respectively). Curves were simultaneously fitted applying the ternary complex model of allosteric interactions according to Ehlert (1988). Given are mean values ± S.E. of two to three experiments carried out as triplicate determination. Scatter bars are not shown when they did not exceed the symbols.

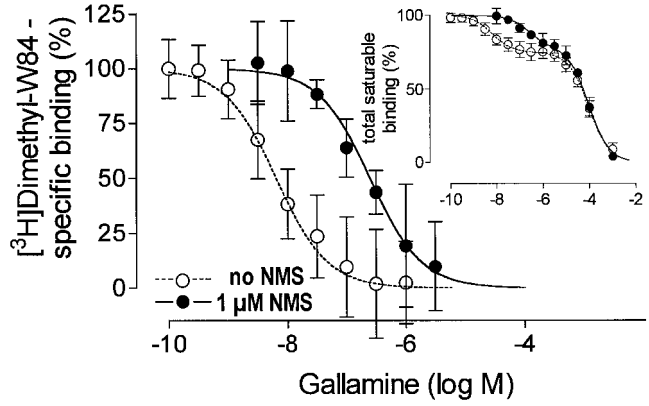

Fig. 2. Inhibition of the saturable binding of [^{3}H]dimethyl-W84 (0.3 nM) by gallamine in free and in NMS-liganded M_2 receptors. Total saturable [^{3}H]dimethyl-W84 binding is depicted in the inset. The component of nonspecific saturable binding was defined as [^{3}H]dimethyl-W84 binding in the presence of 3 μM gallamine. The specific high-affinity fraction is shown enlarged in the main graph. Specific binding at 100% corresponds to a fractional receptor occupancy by [^{3}H]dimethyl-W84 of 0.19 at free receptors, and to 0.14 at NMS-labeled receptors, respectively. The curves were sufficiently described applying a one-site model because the slope factors of the curves did not deviate from unity. Indicated are mean values ± S.E. of three to five experiments carried out in quadruplicate.

rightward shift of the gallamine inhibition curve. The nonspecific low-affinity component of the gallamine displacement curve was not shifted by NMS (Fig. 2, inset). The respective IC_{50} concentrations inducing half-maximum inhibition of specific [^{3}H]dimethyl-W84 binding by gallamine were converted to (−log) K_i values according to Cheng and Prusoff (1973) and amounted to $pK_i = 8.27 \pm 0.39$ (mean ± S.E.; five experiments) at free M_2 receptors and to $pK_i = 6.60 \pm 0.19$ (mean ± S.E.; three experiments), at NMS-occupied receptors. The latter value corresponds favorably with the previously found $pK_i = 6.72$ (Tränkle et al., 1998). The reduction of gallamine affinity by NMS corresponded to a shift in the binding constant of gallamine by a factor of 47. The large scatter bars may raise the question whether the shift is significant. Statistical testing revealed a significant difference between the respective pK_i values (unpaired t test; $P < .03$). Also, the data are better described by two distinct curves than by a single curve through the joint data (F test; $P < .001$).

Figure 3 compiles in the form of a bar graph the binding characteristics of gallamine at free and NMS-occupied M_2 receptors as derived from the experiments with [^{3}H]NMS and with [^{3}H]dimethyl-W84, respectively. The gallamine binding constants for the interaction with free and with NMS-occupied M_2 receptors were independent of whether the radioligand for the orthosteric site or the radioligand for the allosteric site was used. Accordingly, the measured value of the NMS-induced shift of gallamine binding affinity is identical with the shift predicted by the cooperativity model.

In conclusion, the binding characteristics of allosteric agents in G protein-coupled receptors are commonly deduced from alloster effects on the binding of a radioligand for the orthosteric receptor site. Here, the feasibility of the underlying ternary complex model of allosteric interactions was demonstrated by using a new [^{3}H]ligand for direct measurement of alloster binding at muscarinic M_2 receptors.

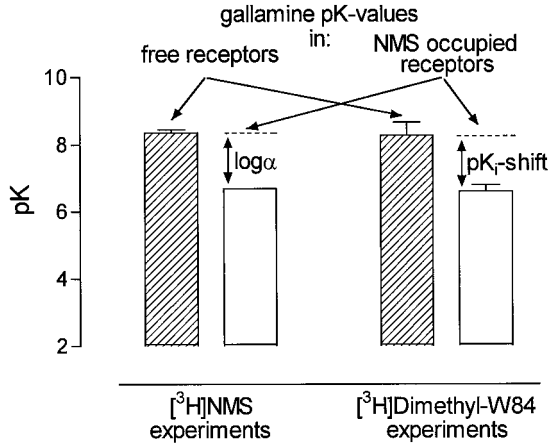

Fig. 3. Binding characteristics of gallamine at free and NMS-occupied M_2 receptors as deduced indirectly from experiments with [^{3}H]NMS applying the cooperativity model (Fig. 1), and derived from competition experiments with the radioalloster [^{3}H]dimethyl-W84 (Fig. 2). Illustrated results are for [^{3}H]NMS experiments: pK_A, −(log) equilibrium dissociation constant of gallamine at free M_2 receptors; α, factor of cooperativity between gallamine and NMS (Ehlert, 1988); according to the ternary complex model, the dissociation constant of gallamine at NMS-occupied receptors is equal to $p(\alpha \cdot K_A)$. Illustrated for [^{3}H]dimethyl-W84 experiments are pK_i, −(log inhibition constant) for the competitive displacement of specific [^{3}H]dimethyl-W84 binding by gallamine at free and at NMS-occupied receptors, respectively. Error bars represent S.E.

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