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Analogs of WIN 62,577 Define a Second Allosteric Site on Muscarinic Receptors

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ABSTRACT

WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole) and WIN 62,577 (17-β-hydroxy-17-α-ethynyl-Δ^4-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole) are potent and centrally active antagonists at rat, but not human, NK_1 receptors. The interactions of these compounds and some analogs with [^{3}H]*N*-methyl scopolamine ([^{3}H]NMS) and unlabeled acetylcholine (ACh) at M_1-M_4 muscarinic receptors have been studied using equilibrium and nonequilibrium radioligand binding methods. The results are consistent with the predictions of the allosteric ternary complex model. The WIN compounds have log affinities for the unliganded receptor in the range 5 to 6.7, and exhibit positive, negative, or neutral cooperativity with [^{3}H]NMS and ACh, depending on the receptor subtype and nature of the interacting ligands. WIN 62,577 is an allosteric enhancer of ACh affinity at M_3 receptors. Although interacting allosterically, WIN 62,577 and WIN 51,708 do not affect [^{3}H]NMS dissociation from M_3 receptors. Certain analogs have higher affinities than WIN 62,577, and truncated forms of WIN 62,577, including steroids, also act allosterically. One analog, 17-β-hydroxy-17-α-Δ^4-androstano[3,2-*b*]pyrido[2,3-*b*]indole (PG987), has the unique effect of speeding [^{3}H]NMS dissociation; its largest effect, 2.5-fold, is at M_3 receptors. The interaction between PG987 and other allosteric agents on [^{3}H]NMS dissociation from M_3 receptors indicate that PG987 binds reversibly to a site distinct from that to which gallamine and strychnine bind: in contrast, PG987 seems to bind to the same site on M_3 receptors as KT5720, staurosporine, and WIN 51,708. Therefore, in addition to the allosteric site that binds strychnine (and probably chloromethyl brucine, another allosteric enhancer) there is a second, nonoverlapping, pharmacologically distinct allosteric site on M_3 receptors that also supports positive cooperativity with ACh.

The five subtypes of muscarinic acetylcholine receptors (M_1–M_5) are members of the superfamily of G-protein coupled receptors (Caulfield and Birdsall, 1998). In addition to the 'primary' sites on the receptor at which agonists and competitive antagonists bind, muscarinic receptors also contain one or more 'allosteric' sites (Ellis, 1997; Holzgrabe and Mohr, 1998; Christopoulos et al., 1998; Christopoulos, 2002; Christopoulos and Kenakin, 2002). The binding of the allosteric ligand to the allosteric site alters the affinity with which muscarinic ligands bind to the primary binding site on the receptor. The cooperative effect of an allosteric agent depends on the particular primary (muscarinic) ligand with which it interacts and may be positive, negative or neutral; if neutral, the allosteric agent binds to the receptor but does not affect the affinity of the primary ligand.

Allosteric agents that enhance the affinity of the endogenous ligand are known as allosteric enhancers, and they may have a number of therapeutic advantages compared with directly acting agonists. These include the possibility of 'absolute' receptor subtype selectivity, independent of the dose or affinity of the allosteric agent, in which the agent shows positive cooperativity with the endogenous ligand at one receptor subtype and neutral cooperativity at the other subtypes (Lazareno and Birdsall, 1995). Allosteric enhancers may have therapeutic utility, for example, in Alzheimer's disease by compensating for the effects of the cholinergic deficit.

We have reported previously that brucine is an allosteric enhancer at M_1 receptors (Lazareno et al., 1998; Birdsall et al., 1999) and that it probably binds to the 'common allosteric site,' which may bind other allosteric ligands, such as gallamine, tubocurarine, obidoxime, strychnine, and Me-WDuo (Ellis and Seidenberg, 1992, 2000; Waelbroeck, 1994; Proska and Tucek, 1995; Tränkle and Mohr, 1997). Recently, we reported that KT5720, a staurosporine analog, is also an allosteric enhancer at M_1 receptors, but it binds to a site that

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ABBREVIATIONS: KT5720, (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid hexyl ester; WIN 51,708, 17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole; WIN 62,577, 17-β-hydroxy-17-α-ethynyl-Δ^4-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole; NMS, *N*-methyl scopolamine; ACh, acetylcholine; PG987, 17-β-hydroxy-17-α-Δ^4-androstano[3,2-*b*]pyrido[2,3-*b*]indole; QNB, 3-quinuclidinylbenzilate.

is different from the 'common allosteric site' (Lazareno et al., 2000).

In our search for allosteric enhancers, we have found that WIN 51,708 and WIN 62,577 (Fig. 1) act allosterically at muscarinic receptors and that WIN 62,577 is an allosteric enhancer at M_3 receptors. These compounds are antagonists of rat, but not human, NK_1 receptors and, most importantly, they are centrally active. A number of analogs were therefore synthesized and here we describe the allosteric interactions of the WIN compounds and analogs with [^{3}H]NMS and ACh at M_1-M_4 receptors. Most of the active compounds, in common with all known allosteric agents at muscarinic receptors, inhibit to some degree the dissociation of [^{3}H]NMS. One compound, **3** (PG987), however, has the unique effect of speeding [^{3}H]NMS dissociation, especially at M_3 receptors. This characteristic has allowed us to determine that at M_3 receptors, this compound binds to a different site from the 'common allosteric site'.

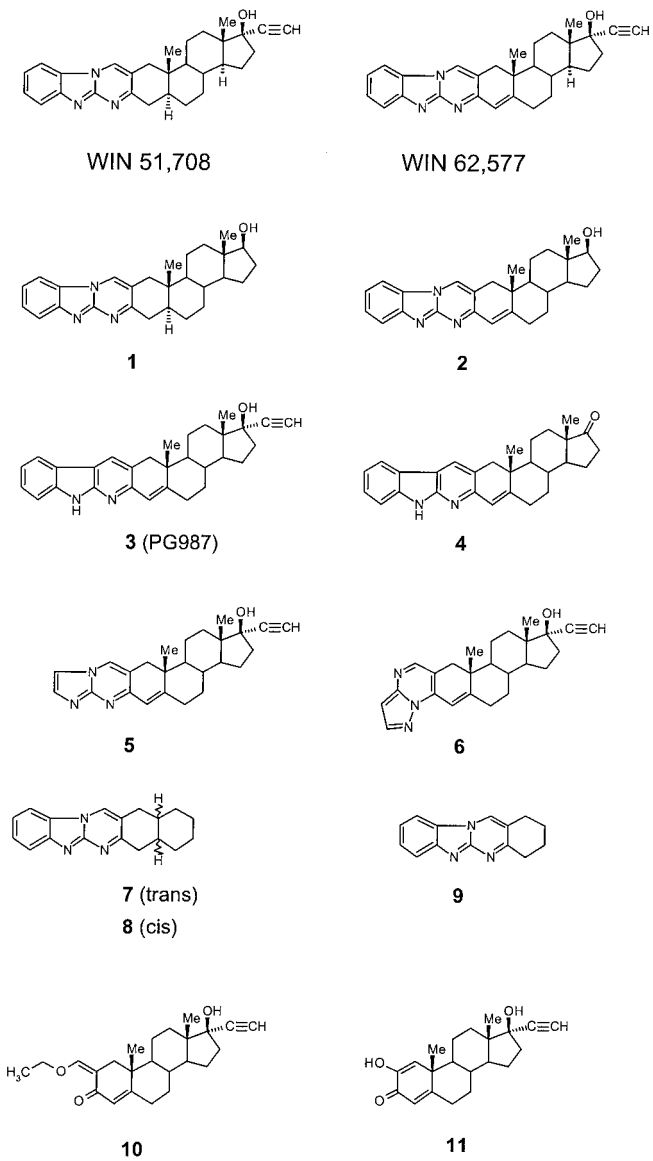

Fig. 1. Structures of WIN 51,708, WIN 62,577, and analogs. **7** and **8** have a *trans* and *cis* configuration, respectively, and are both racemates.

Materials and Methods

Materials. [^{3}H]NMS (81–86 Ci/mmol) was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Strychnine HCl, gallamine triiodide, and ACh chloride were from Sigma Chemical Co. (Dorset, UK). Staurosporine was from Sigma and from Alexis Corporation (Nottingham, UK), and KT5720 was from Alexis, TCS Biologicals Ltd (Buckingham, UK), and Calbiochem (Nottingham, UK). WIN 51,708 and WIN 62,577 were from Sigma/RBI (Gillingham, UK).

Cell Culture and Membrane Preparation. Chinese hamster ovary cells stably expressing cDNA encoding human muscarinic M_1–M_4 receptors (Buckley et al., 1989) were generously provided by Dr. N. J. Buckley (University of Leeds, Leeds, UK). These were grown in α-minimal essential medium (Invitrogen, Paisley, UK) containing 10% (v/v) newborn calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine, at 37°C under 5% CO_2. Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM HEPES and 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) followed by centrifugation (40,000*g*, 15 min), were washed once in 20 mM HEPES and 0.1 mM EDTA, pH 7.4, and were stored at −70°C in the same buffer at protein concentrations of 2 to 5 mg/ml. Protein concentrations were measured with the Bio-Rad (Hemel Hempstead, UK) reagent using bovine serum albumin as the standard. The yields of receptor varied from batch to batch but were approximately 10, 1, 2, and 2 pmol/mg of total membrane protein for the M_1, M_2, M_3, and M_4 subtypes, respectively.

Radioligand Binding Assays. Unless otherwise stated, frozen membranes were thawed, resuspended in incubation buffer containing 20 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4, and incubated with radioligand and unlabeled drugs for 2 h at 30°C in a volume of 1 ml. Membranes were collected by filtration over glass fiber filters (Whatman GF/B) presoaked in 0.1% polyethylenimine, using a Brandel cell harvester (Semat, St.-Albans, UK), extracted overnight in scintillation fluid (ReadySafe; Beckman Coulter, High Wycombe, UK), and counted for radioactivity in Beckman Coulter LS6000 scintillation counters. Membrane protein concentrations (5–50 μg/ml) were adjusted so that not more than about 15% of added radioligand was bound. Nonspecific binding was measured in the presence of 10^{-6} M 3-quinuclidinylbenzilate (QNB; an antagonist with picomolar potency) and accounted for 1 to 5% of total binding. GTP was present at a concentration of 2×10^{-4} M in assays containing unlabeled ACh. Data points were usually measured in duplicate. Chinese hamster ovary cell membranes do not possess cholinesterase activity (Lazareno and Birdsall, 1993; Gnagey and Ellis, 1996) so ACh could be used in the absence of a cholinesterase inhibitor. The test compounds were dissolved in dimethyl sulfoxide, which, at the highest final concentration of 2%, had no effect on binding.

Data Analysis. General data preprocessing, as well as the 'affinity ratio' calculations and routine plots of the semiquantitative equilibrium assay, were performed using Minitab (Minitab Ltd, Coventry, UK). The other assays were analyzed with nonlinear regression analysis using the fitting procedure in SigmaPlot (SPSS Inc., Erkrath, Germany). This procedure is relatively powerful in that it allows the use of two or more independent variables (e.g., concentrations of two drugs).

Equilibrium Binding Assays for Estimation of the Affinity of an Allosteric Agent for the Receptor and the Magnitude of Its Cooperativity with [^{3}H]NMS and ACh. The design and analyses have been described in detail (Lazareno and Birdsall, 1995; Lazareno et al., 1998). Briefly, specific binding of a low concentration of [^{3}H]NMS (1–2 times the K_d) was measured in the presence of a number of concentrations of test agent, all in the absence and presence of one or more concentrations of ACh. Specific binding of a high

concentration of [^{3}H]NMS (5–10 times K_d) was also measured. Nonlinear regression analysis was used to fit the data to the equation

$$B_{LAX} = \frac{B_{max} \times L \times K_L \times (1 + \alpha \times (X \times K_X)^s)}{1 + (X \times K_X)^s + (A \times K_A)^n \times (1 + \beta \times (X \times K_X)^s X + L \times K_L \times (1 + \alpha \times (X \times K_X)^S)} \quad (1)$$

where B_{LAX} is observed specific bound radioligand; L, A, and X are concentrations of [^{3}H]NMS, ACh, and allosteric agent, respectively; K_L, K_A, and K_X are affinity constants for the corresponding ligands and the receptor, α and β are allosteric constants of X with [^{3}H]NMS and ACh, respectively; n is a logistic slope factor to describe the binding of ACh, and s is a 'Schild slope' factor to describe the binding of X. According to the allosteric model, s should be 1.

Above a certain concentration, some allosteric agents, especially those that exhibit neutral or positive cooperativity with [^{3}H]NMS, may slow the kinetics of [^{3}H]NMS binding so much that the binding does not reach equilibrium. In most cases, sufficient incubation time was used to allow [^{3}H]NMS binding in the presence of the agent to reach equilibrium. In a few cases, however, the highest concentration of agent would be predicted to slow [^{3}H]NMS kinetics sufficiently to prevent binding equilibrium from being reached; in these cases, the data were better fitted to the equation

$$B_{LAXt} = B_{LAX} + (B_{L_0} - B_{LAX}) \times \left(\exp\left(\frac{-t \times k_{off}}{1 + \alpha \times (X \times K_X)^s} + \frac{-t \times k_{off} \times L \times K_L}{1 + (X \times K_X)^s + (A \times K_A)^n \times (1 + \beta \times (X \times K_X)^s)} \right) \right) \quad (2)$$

where B_{LAXt} is observed specific binding under nonequilibrium conditions, B_{LAX} is the predicted equilibrium binding defined in eq. 1, t is the incubation time, k_{off} is the dissociation rate constant of [^{3}H]NMS, and B_{L_0} is the initial amount of bound radioligand, set to zero in this case. This equation assumes that the dissociation of [^{3}H]NMS from the allosteric agent-occupied receptor is negligible, and that the binding kinetics of both ACh and the allosteric agent are fast in comparison with the dissociation rate of [^{3}H]NMS.

If only a single concentration of ACh was used, the data were visualized with 'affinity ratio' plots, where the affinity ratio is the apparent affinity of the 'primary' ligand ([^{3}H]NMS or ACh) in the presence of a particular concentration of test agent divided by the apparent affinity of the primary ligand in the absence of test agent. Theoretically, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the K_d of the test agent at the free receptor, and the asymptotic level corresponds to the cooperativity constant for the test agent and primary ligand (Lazareno and Birdsall, 1995). Affinity ratios were calculated from the specific binding data as follows (Lazareno et al., 1998).

The B_{max} was calculated using

$$B_{max} = \frac{B_{L1} \times B_L \times (L - L_1)}{L \times B_{L_1} - L_1 \times B_L} \quad (3)$$

The apparent affinity, K_{LX}, of [^{3}H]NMS in the presence of a particular concentration of test agent was estimated using

$$K_{LX} = \frac{B_{LX}}{L \times (B_{max} - B_{LX})} \quad (4)$$

The apparent affinity, K_{AX}, of ACh in the presence of the test agent alone was estimated using

$$K_{AX} = \frac{B_{max} \times (B_{LX} - B_{LAX})}{A \times B_{LAX} \times (B_{max} - B_{LX})} \quad (5)$$

where B_L is binding in the presence of the low [[^{3}H]NMS], L, alone; B_{L1} is binding in the presence of the high [[^{3}H]NMS], L_1; B_{LA} is binding in the presence of the low [[^{3}H]NMS] and ACh; B_{LX} is binding in the presence of the low [[^{3}H]NMS] and a particular concentration of test agent; B_{LAX} is binding in the presence of the low [[^{3}H]NMS], ACh, and the same concentration of test agent.

These apparent affinities, K_{LX} and K_{AX}, were divided by the 'true' affinities K_L and K_A [measured in the absence of test agent (i.e., where $B_{LX} = B_L$ and $B_{LAX} = B_{LA}$)] to obtain the affinity ratios of [^{3}H]NMS and ACh.

Off-Rate Assay to Estimate the Affinity of an Allosteric Agent for the [^{3}H]NMS-Occupied Receptor. A high concentration of membranes (2–4 mg protein/ml) was incubated with a high concentration of [^{3}H]NMS (5 nM) for about 15 min. Then 10-μl aliquots were distributed to tubes that were empty or contained 1 ml of 10^{-6} M QNB alone and in the presence of a number of concentrations of allosteric agent (typically $n = 4$). Nonspecific binding was measured in separately prepared tubes containing 10 μl of membrane and 2 μl of [^{3}H]NMS + QNB. Some time later (about 2.5 dissociation half-lives), the samples were filtered. The data were transformed to observed rate constants, k_{offobs}, using the formula

$$k_{offobs} = \ln(B_0/B_t)/t \quad (6)$$

where B_0 is initially bound radioligand and B_t is bound radioligand remaining after t min of dissociation. These values were expressed as percentage of the true [^{3}H]NMS dissociation rate constant k_{off} (k_{offobs} in the absence of allosteric agent) and fitted to a logistic function using nonlinear regression analysis. Theoretically, the curves should have slopes of 1 and correspond to the occupancy curves of the allosteric agents at the [^{3}H]NMS-occupied receptors, regardless of whether the change of [^{3}H]NMS dissociation is caused by an allosteric change in the shape of the receptor or the trapping of the [^{3}H]NMS in its binding pocket by the bound allosteric agent (Lazareno and Birdsall, 1995). Initially the curve was fitted without constraints. If the slope factor was not different from 1, and the maximal effect (E_{max}) was not less than zero, then the slope was constrained to 1 and the E_{max} was fitted. If the fitted E_{max} was less than zero (a physical impossibility, apart from experimental variation or error) then the E_{max} was constrained to zero and the slope fitted. With the compounds under study, the E_{max} was often greater than zero, and in most such cases, the data were well fitted with the slope constrained to 1.

In off-rate assays to study the interaction between two compounds, the data were transformed to values of k_{offobs} as described above and fitted to the following equation (Lazareno et al., 2000):

$$k_{offobs} = k_{off} \frac{1 + X \times K_{Xo} \times \rho_X + Y \times K_{Yo} \times (\rho_Y + \rho_{XY} \times K_{Xo} \times \delta \times X)}{1 + X \times K_{Xo} + Y \times K_{Yo} \times (1 + X \times \delta \times K_{Xo})} \quad (7)$$

where k_{off} is the dissociation rate constant of [^{3}H]NMS from the unliganded receptor, X and Y are the concentrations of two allosteric agents, and K_{Xo} and K_{Yo} are the affinity values of X and Y, respectively for the [^{3}H]NMS-occupied receptor. δ Is the cooperativity between X and Y, so that if $\delta = 0$, the interaction is apparently competitive, and if $\delta = 1$, the compounds are neutrally cooperative or noninteracting. ρ_X, ρ_Y, and ρ_{XY} are the dissociation rate constants of [^{3}H]NMS from the receptor occupied by X, by Y, and by both X and Y, respectively, expressed as a fraction of k_{off}.

Chemistry. Compounds **1** to **11** were synthesized by Sankyo Co Ltd, Tokyo, Japan. The general synthetic procedure used (Bajwa and Sykes, 1980a,b; Venepalli et al., 1992) involves the condensation of an aminoheterocycle (e.g., 2-aminobenzimidazole, 2-aminoimidazole, 2-aminoindole) with an α-hydroxymethylene ketone**,** prepared from the ketone, usually a 3-keto steroid (but also *trans*- and *cis*-decalone and -cyclohexanone for **7** to **9**, respectively) by standard formylation procedures (Bajwa and Sykes, 1980a) to yield the compounds shown in Fig. 1. Compounds **1**, **2**, and **5** are reported in Bajwa and Sykes (1980a) and **9** is described in Bajwa and Sykes (1979) and Venepalli et al. (1992). **7** and **8** are racemates. Some additional steroid derivatives, **10** and **11**, were isolated as side products or intermediates

and were also assayed for activity. All compounds were characterized by high-performance liquid chromatography (> 99% purity), elemental analysis, NMR, and mass spectrometry.

Results

The Allosteric Properties of WIN 51,708 and WIN 62,577. The easiest way to detect and quantify an allosteric interaction with muscarinic receptors is to measure the effect of the agent on the dissociation rate of the radiolabeled antagonist [^{3}H]NMS. We achieve this by measuring the [^{3}H]NMS dissociated from the receptor at a single time point, alone, and in the presence of a range of concentrations of test agent. The data points are converted to rate constants and expressed as a percentage of control off-rate, as described under *Materials and Methods*. Theoretically, the curves should have slopes of 1, and reflect the occupancy curves of the allosteric agents at the [^{3}H]NMS-occupied receptors.

WIN 51,708 potently and strongly inhibited [^{3}H]NMS dissociation at M_2 and M_4 receptors, exhibiting about 10-fold M_4 selectivity (Fig. 2A). It caused a submaximal inhibition at the M_1 receptor and had no effect at the M_3 receptor. WIN 62,577, a close analog of WIN 51,708 but containing a double bond in the steroid moiety (Fig. 1), was about 10-fold weaker at inhibiting dissociation from the [^{3}H]NMS-occupied M_4 receptor and had a smaller maximal effect at this subtype as well as at M_2 receptors. WIN 62,577, like WIN 51,708, had no measurable effect on [^{3}H]NMS dissociation from M_3 receptors. The parameter estimates, describing the slowing effects of these two ligands and the other compounds examined, are summarized in Table 1. These kinetic studies provide evidence that the two WIN compounds have an allosteric action at M_1, M_2, and M_4 receptors. There is no evidence from these data for an allosteric action at M_3 receptors (but see *Interactions among Allosteric Agents*).

To characterize these interactions further, the equilibrium effects of the two WIN compounds on equilibrium [^{3}H]NMS and ACh binding were measured. To determine the allosteric effects on ACh affinity, we used an indirect assay of ACh binding that measures the inhibition of [^{3}H]NMS binding by unlabeled ACh. This is because [^{3}H]ACh does not usefully bind to M_1 or M_3 receptors; in any case, GTP is included in the assay to minimize the effect of G-protein coupling on the affinity of ACh. The effects of a range of concentrations of test agent on [^{3}H]NMS binding are measured in the absence and presence of one or more concentrations of ACh. The allosteric effects on [^{3}H]NMS and ACh binding are disentangled in two ways. For quantitative analysis, the data are fitted to eq. 1 or 2 as appropriate. If a single concentration of ACh is used, then the data are transformed to 'affinity ratios' and plotted against log [test agent] (see *Materials and Methods*). In theory, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the K_d of the test agent at the free receptor, and the asymptotic level corresponds to the cooperativity constant for the test agent and primary ligand (Lazareno and Birdsall, 1995)

Figure 2B shows representative affinity ratio plots from equilibrium assays for WIN 51,708 and WIN 62,577 binding to M_1–M_4 receptors. The parameter estimates from nonlinear regression analysis for these compounds and the others examined in this article are summarized in Table 1. WIN 51,708 showed a small degree of positive cooperativity with [^{3}H]NMS at M_2 receptors and a larger positive effect at M_4 receptors. The inhibitory effect of $\geq 10^{-5}$ M WIN 51,708 on [^{3}H]NMS binding to M_4 receptors is caused by the strong slowing effect of these high concentrations on [^{3}H]NMS association and is accounted for by eq. 2 (see *Materials and Methods*). In contrast, WIN 62,577, which was up to 5-fold less potent, showed only small negative cooperativity with [^{3}H]NMS at all subtypes.

With respect to ACh, WIN 51,708 showed small negative cooperativity at M_1 and M_3 receptors and larger negative cooperativity at M_2 and M_4 receptors, whereas WIN 62,577 was also negative at M_2 and M_4 receptors, almost neutral at M_1 receptors, and showed a small but nonsignificant positive interaction with ACh at M_3 receptors (1.8 ± 0.5-fold, $n = 4$). This positive cooperativity was confirmed in more detailed assays (Fig. 2C), where full [^{3}H]NMS-ACh curves were constructed in the absence and presence of three concentrations of WIN 62,577.

There is an excellent compatibility between the affinity estimates for the WIN compounds at the[^{3}H]NMS-occupied M_1, M_2, and M_4 receptors measured directly in the kinetic assay and the corresponding values derived from equilibrium assays (Table 1). It is worth noting at this point that the potency and small degree of negative cooperativity with [^{3}H]NMS of both WIN compounds at M_3 receptors in equilibrium assays would predict some activity at M_3 receptors in the off-rate assay, but no activity was observed.

Allosteric Properties of Analogs of WIN 51,708 and WIN 62,577. A number of analogs of WIN 51,708 and WIN 62,577 were synthesized in which both the heterocyclic moiety and the attached alicyclic ring systems were modified (Fig. 1). The allosteric properties of these analogs in the off-rate and equilibrium assays are illustrated in Figs. 3 and 4, respectively. The estimated affinities of the compounds and their cooperativities with ACh and NMS are shown in Table 1, which illustrates the excellent agreement between the kinetic and equilibrium data and the predictions of the allosteric ternary complex model.

The ethynyl substituent of WIN 51,708 and WIN 62,577 does not seem to be required for high-affinity binding because the corresponding 17-hydroxy analogs, **1** and **2**, have comparable or higher affinities. In fact, the log affinity of 7.0 for **1** at the free M_4 receptor and its positive cooperativity with [^{3}H]NMS represents the most potent interaction observed in this study. This compound also exhibited the greatest subtype selectivity—up to 50-fold higher affinity for the M_4 versus M_3 receptor. Compound **4**, a 17-keto steroid analog of **3**, is inactive, which suggests the 17-hydroxyl group may be important for binding to the receptor.

Because WIN 51,708 and WIN 62,577 are very extended structures, attempts were made to determine whether there were minimal substructures of these compounds that supported allosterism. The allosteric interactions are maintained in **3**, **5**, and **6**, where the heterocyclic ring system of WIN 62,577 has been modified or truncated. A large (8-fold) positive cooperativity with [^{3}H]NMS is found for **6** acting at M_1 receptors. Similarly, analogs **8** and **9**, in which the steroid moiety has been truncated, are allosteric ligands but are 10- to 100-fold weaker than WIN 51,708. The *trans*-decalin analog **7** was inactive, as were further truncated analogs (data not shown). Finally, two steroid structures, **10** and **11**, corresponding to the steroid portion of the active compounds WIN 62,577, **3**, **5**, and **6,** show surprising activity in both the

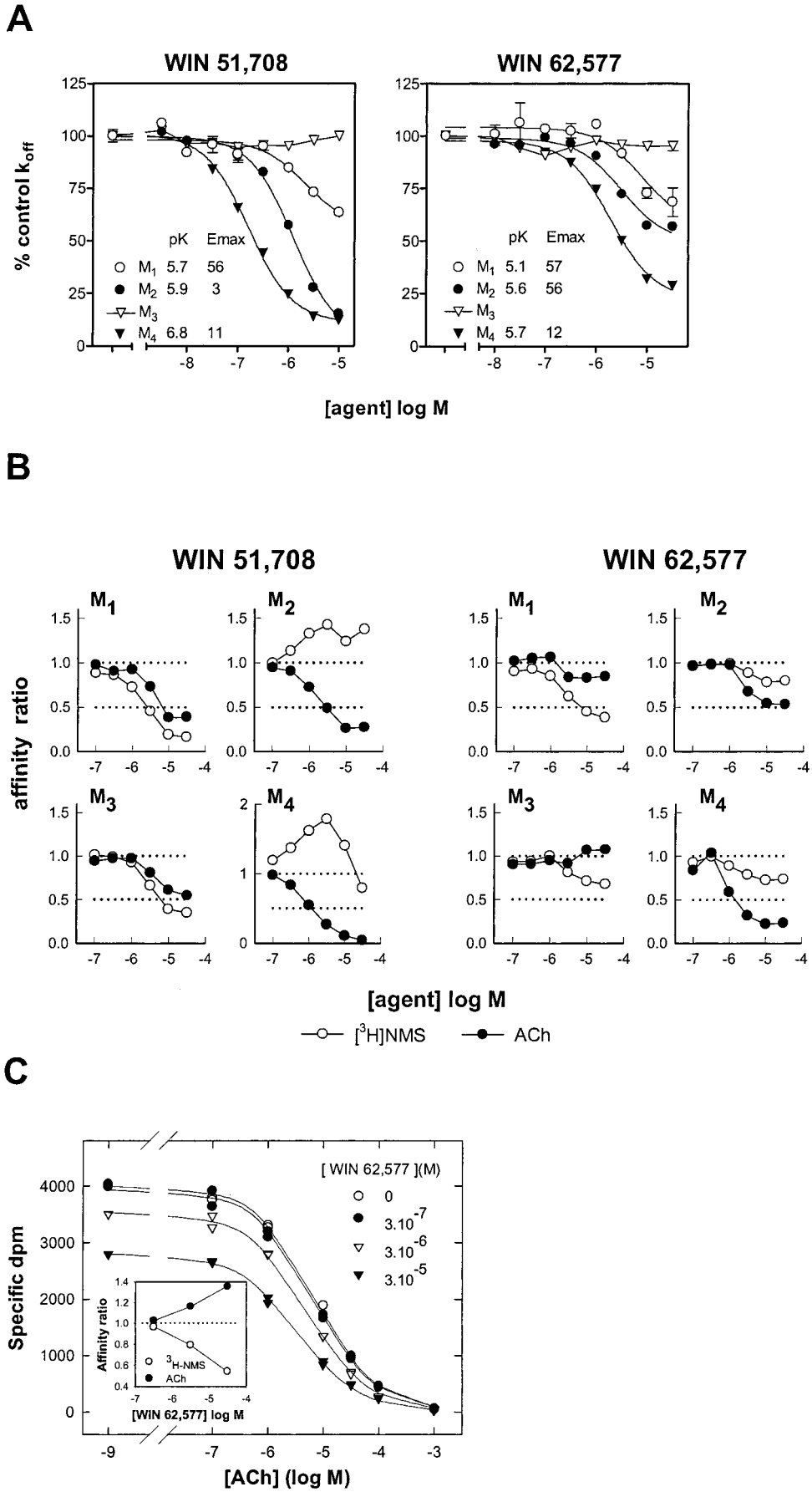

Fig. 2. A, concentration-dependent effects of WIN 51,708 and WIN 62,577 on the [^{3}H]NMS-occupied receptor. The dissociation rate constant of [^{3}H]NMS from M_1–M_4 receptors was measured in duplicate at a single time point and expressed as percentage of control, as described under Materials and Methods. The legends indicate the EC_{50} values obtained using nonlinear regression analysis of these curves to a logistic function. B, concentration-dependent effects of WIN 51,708 and WIN 62,577 on the equilibrium binding of [^{3}H]NMS and ACh. The effects are expressed as 'affinity ratios' [i.e., the apparent affinity of the 'primary' ligand ([^{3}H]NMS or ACh) in the presence of a particular concentration of test agent divided by its apparent affinity in the absence of test agent]. Affinity ratios were calculated as described in under *Materials and Methods*. Affinity ratios >1 indicate positive cooperativity, affinity ratios <1 indicate negative cooperativity, and affinity ratios of 1 with one primary ligand at concentrations of test agent that modify the binding of the other primary ligand indicate neutral cooperativity, The IC_{50}, or EC_{50}, of a test agent on the affinity ratio of either primary ligand corresponds approximately to the K_d of the test agent for the free receptor. High concentrations of compounds that show neutral or positive cooperativity with [^{3}H]NMS and that strongly inhibit [^{3}H]NMS dissociation may inhibit [^{3}H]NMS binding through a kinetic effect (i.e., lack of equilibration of [^{3}H]NMS binding; for example, WIN 51,708 at M_4 sites). C, inhibition of [^{3}H]NMS binding to M_3 receptors by ACh, alone and in the presence of three concentrations of WIN 62,577. GTP (200 μM) was present. The data were fitted to eq. 1 (see *Materials and Methods*) and the inset shows affinity ratios (1/dose ratio) for ACh and [^{3}H]NMS, calculated from the parameters of the fit. A second assay gave almost identical results, and from both assays (n = 2) the log affinity of WIN 62,577 was 5.21 ± 0.10, cooperativity with [^{3}H]NMS was 0.42 ± 0.06, and cooperativity with ACh was 1.39 ± 0.03.

off-rate and equilibrium assays (Fig. 5), including positive cooperativity with [^{3}H]NMS. The data suggest that the low-molecular-weight analogs **9** and **11** are capable of exerting their allosteric actions by binding to distinct subsites of the allosteric site labeled by WIN 51,708 and WIN 62,577.

The most surprising result in the studies reported in this article is that in the off-rate assay, **3** (also called PG987) had the unique effect of speeding up [^{3}H]NMS dissociation. It caused a 2- to 3-fold increase in [^{3}H]NMS off-rate at M_3 receptors, with smaller effects at the other subtypes; the order of effectiveness was $M_3 > M_1 > M_4 > M_2$ (Fig. 3). This effect was confirmed in full dissociation assays (Fig. 6), which also indicated that the dissociation of [^{3}H]NMS was monoexponential at all subtypes, both in the absence and presence of PG987 (Fig. 6, insets).

In view of the unusual effects of PG987 on [^{3}H]NMS dissociation kinetics, we explored in more detail the interaction between PG987 and [^{3}H]NMS binding at M_3 receptors. PG987 increased the association rate of [^{3}H]NMS, but less than it did the dissociation rate, thus accounting quantitatively for the observed reduction in the affinity of [^{3}H]NMS (Fig. 7A). The partial inhibition of [^{3}H]NMS binding by 10 μM PG987 reflected mainly a reduction of apparent affinity, although a small reduction of B_{max} was also sometimes observed (Fig. 7B). Reversibility of the effects of PG987 on [^{3}H]NMS binding was studied by preincubating membranes with PG987 at 10 times

TABLE 1

Parameter estimates from equilibrium and off-rate assays

Values are reported as mean ± S.E. (when $n = 2$ the S.E. is equal to range/2): if a standard error is missing, then only a single estimate could be made. From the equilibrium assay, K is the affinity of the compound for the free receptor. K_{NMSocc} is an estimate of the affinity of the compound at the [^{3}H]NMS-occupied receptor and is the product of K and the cooperativity with [^{3}H]NMS: K_{NMSocc} was not estimated if the cooperativity with [^{3}H]NMS was less than 0.05. In the off-rate assay, the E_{max} is expressed as percentage of control [^{3}H]NMS dissociation rate constant. $-\log EC_{50}$ is a second, independent estimate of the log affinity of the compound at the [^{3}H]NMS-occupied receptor, and the final column compares the two log affinity estimates: values of ±0.5 (3-fold difference) or less indicate that the results from the two types of assay are internally consistent with the ternary complex allosteric model. This comparison was made only if there were at least two estimates for each measure. The slope factors in both the equilibrium and off-rate assays were generally not different from one, with the following exceptions: **5** had slopes of 1.4 to 1.8 in the off-rate assay, **8** had slopes of 1.2 to 1.9 in the equilibrium assay, and **9** had slopes of 1.3 to 2 in both the equilibrium and off-rate assays.

		Equilibrium Assay					Offrate Assay			
			Cooperativity							$-\log EC_{50}$
Name	Subtype	$\log K$	NMS	ACh	$\log K_{NMSocc}$	n	$-\log EC_{50}$	E_{max} %	n	$-\log K_{NMSocc}$
		M^{-1}					M			
WIN	M_1	5.8 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	5.1 ± 0.1	4	5.6 ± 0.1	50 ± 4	3	0.4
51708	M_2	5.9 ± 0.1	1.7 ± 0.2	0.2 ± 0.0	6.2 ± 0.1	3	5.9 ± 0.0	5 ± 1	3	–0.2
	M_3	5.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	5.1 ± 0.2	3	N.D.			
	M_4	6.2 ± 0.0	3.1 ± 0.2	0.0 ± 0.0	6.7 ± 0.1	3	6.8 ± 0.1	4 ± 4	3	0.1
WIN	M_1	5.5 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	5.0 ± 0.0	4	5.0 ± 0.2	35 ± 23	3	0.0
62577	M_2	5.3 ± 0.3	0.6 ± 0.1	0.2 ± 0.1	5.0 ± 0.4	3	5.3 ± 0.1	34 ± 11	3	0.3
	M_3	5.1 ± 0.2	0.4 ± 0.1	1.8 ± 0.5	5.0 ± 0.2	4	N.D.			
	M_4	5.9 ± 0.2	0.8 ± 0.1	0.1 ± 0.0	5.8 ± 0.2	3	5.6 ± 0.1	21 ± 4	3	–0.2
1	M_1	5.7 ± 0.2	1.7 ± 0.3	0.3 ± 0.1	5.9 ± 0.2	3	5.9 ± 0.1	8 ± 4	3	–0.1
	M_2	6.6 ± 0.2	1.3 ± 0.1	0.4 ± 0.1	6.7 ± 0.2	3	6.1 ± 0.0	–2 ± 2	3	–0.6
	M_3	5.3 ± 0.3	1.8 ± 0.3	0.4 ± 0.1	5.5 ± 0.2	3	6.1 ± 0.1	70 ± 6	2	0.6
	M_4	7.0 ± 0.1	2.2 ± 0.2	0.2 ± 0.1	7.4 ± 0.2	3	7.0 ± 0.0	–2 ± 3	3	–0.4
2	M_1	5.7 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	5.5 ± 0.1	3	5.3 ± 0.1	11 ± 2	3	–0.1
	M_2	5.6 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	5.4 ± 0.2	3	5.6 ± 0.0	7 ± 3	3	0.2
	M_3	4.9 ± 0.4	0.4 ± 0.2	1.3 ± 0.2	4.9 ± 0.4	3	5.2	91	1	
	M_4	5.9 ± 0.1	1.6 ± 0.4	0.1 ± 0.0	6.1 ± 0.1	3	6.2 ± 0.0	3 ± 1	3	0.1
3	M_1	6.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	5.9 ± 0.1	4	6.1 ± 0.0	192 ± 11	3	0.2
	M_2	6.3 ± 0.1	0.6 ± 0.0	0.1 ± 0.0	6.0 ± 0.1	4	5.8 ± 0.2	134 ± 12	3	–0.2
	M_3	6.6 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	6.1 ± 0.0	3	6.1 ± 0.1	246 ± 18	4	0.0
	M_4	6.1 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	5.8 ± 0.1	3	6.0 ± 0.1	166 ± 9	3	0.3
5	M_1	4.8 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	4.7	2	4.7 ± 0.0	–2 ± 2	3	
	M_2	4.8 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	4.3 ± 0.0	2	4.3 ± 0.1	0 ± 0	3	0.1
	M_3	4.5 ± 0.1	0.0 ± 0.0	0.1 ± 0.0		2	3.5 ± 0.0	0 ± 0	3	
	M_4	5.0 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	4.4 ± 0.3	2	4.6 ± 0.1	0 ± 0	3	0.2
6	M_1	6.0 ± 0.1	8.1 ± 1.5	0.4 ± 0.1	6.9 ± 0.1	3	6.9 ± 0.0	–1 ± 1	3	–0.1
	M_2	6.6 ± 0.1	1.4 ± 0.1	0.5 ± 0.0	6.7 ± 0.1	3	6.2 ± 0.1	5 ± 2	3	–0.5
	M_3	6.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	5.8 ± 0.2	3	6.7 ± 0.2	89 ± 1	3	0.9
	M_4	6.1 ± 0.0	1.5 ± 0.1	0.4 ± 0.0	6.3 ± 0.1	3	6.3 ± 0.1	9 ± 3	3	0.0
8	M_1	5.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	4.2	2	4.7 ± 0.1	0 ± 0	2	
	M_2	5.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	4.3	2	4.5 ± 0.1	–1 ± 1	2	
	M_3	5.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1		2	4.7 ± 0.0	51 ± 4	2	
	M_4	5.5± 0.0	0.0 ± 0.0	0.0 ± 0.0		2	4.7 ± 0.1	12 ± 15	2	
9	M_1	4.7 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	4.6 ± 0.1	2	4.8 ± 0.0	0 ± 0	3	0.2
	M_2	4.5 ± 0.1	1.7 ± 0.2	0.0 ± 0.0	4.7 ± 0.0	2	4.8 ± 0.0	–4 ± 2	3	0.1
	M_3	4.8 ± 0.1	0.6 ± 0.1	0.0 ± 0.0	4.6 ± 0.1	2	4.6 ± 0.0	–1 ± 1	3	0.0
	M_4	4.6 ± 0.0	1.2 ± 0.1	0.0 ± 0.0	4.7 ± 0.1	2	4.8 ± 0.0	–1 ± 2	3	0.1
10	M_1	4.6	0.0	0.2		1	5.2	69	1	
	M_2	5.0	0.9	0.4	4.9	1	4.3	0		
	M_3	3.9	0.0	0.2		1	4.9	60	1	
	M_4	4.6	1.5	0.0	4.7	1	5.0	9	1	
11	M_1	4.3 ± 0.1	0.0 ± 0.0	0.4 ± 0.1		2	N.D.			
	M_2	5.0 ± 0.4	1.0 ± 0.0	0.6 ± 0.1	5.0 ± 0.3	2	4.3 ± 0.1	6 ± 10	3	–0.6
	M_3	4.1 ± 0.2	0.3 ± 0.3	0.6 ± 0.0	4.1	2	5.3 ± 0.6	87 ± 2	3	
	M_4	4.5 ± 0.1	0.8 ± 0.1	0.1 ± 0.1	4.3 ± 0.2	2	4.2 ± 0.0	0 ± 0	3	–0.1

N.D., could not be determined.

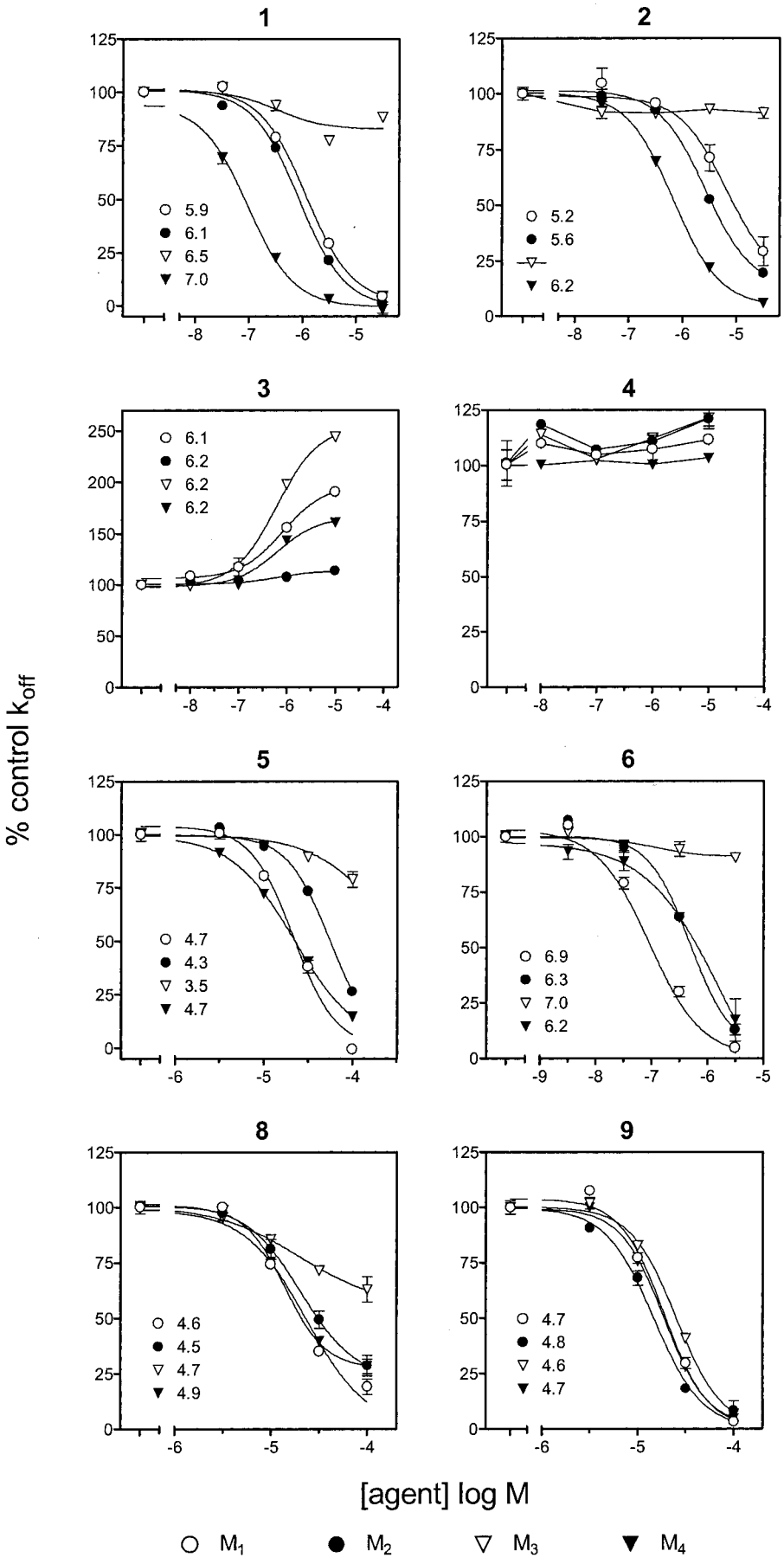

Fig. 3. Effects of WIN 62,577 analogs on [^{3}H]NMS dissociation. For more information, see the legend to Fig. 2A.

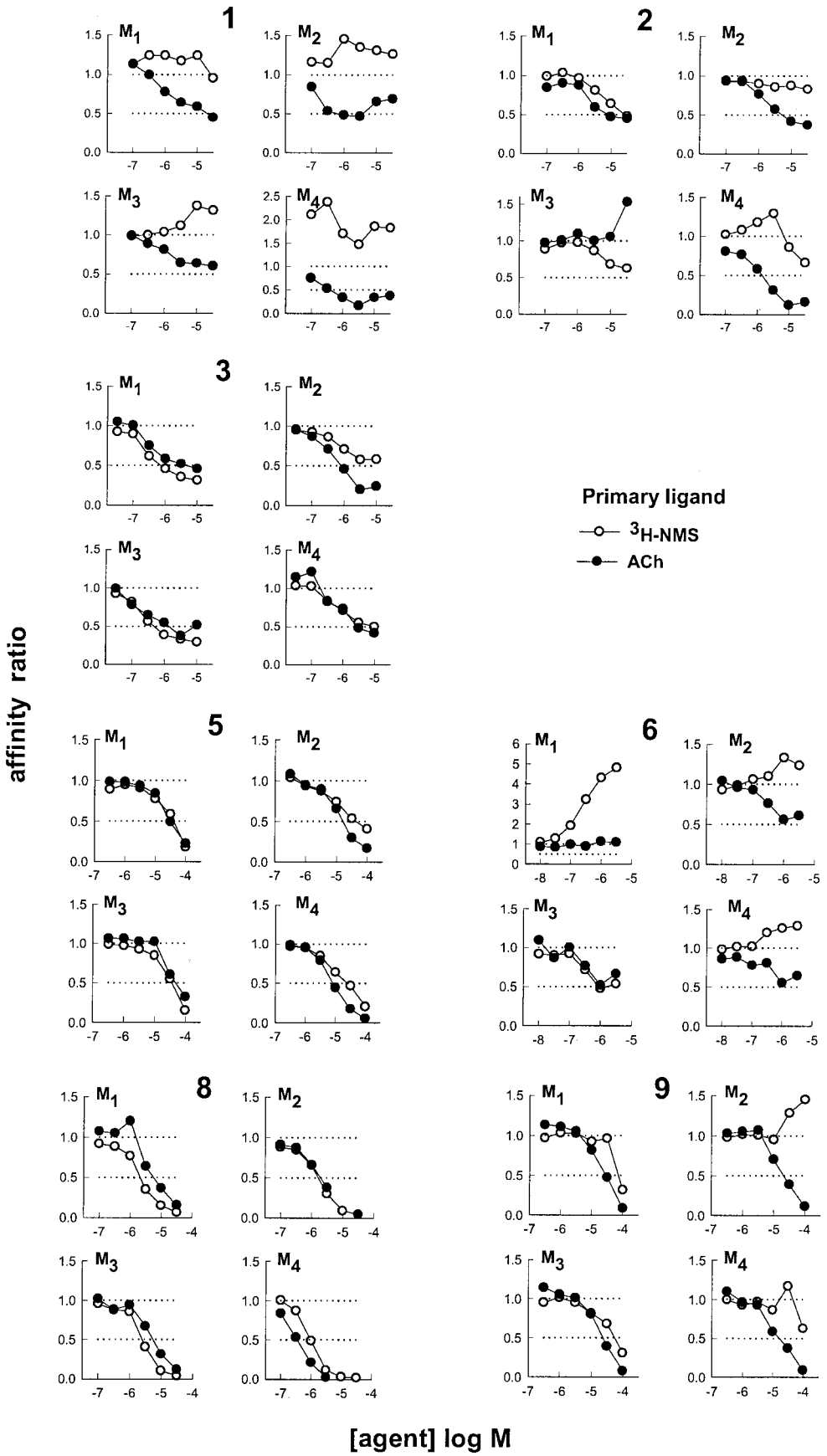

Fig. 4. Effects of WIN 62,577 analogs on the equilibrium binding of [^{3}H]NMS and ACh. For more information, see the legend to Fig. 2B.

the final concentration; such preincubation did not affect the potency of PG987 after subsequent dilution but caused small and variable increases in the inhibitory effect (Fig. 7C). These results suggest that the binding of PG987 to the receptor is reversible, but high concentrations of PG987 may perturb the membrane environment in some way that sometimes leads to small reductions in binding capacity.

Interactions among Allosteric Ligands. The effect of PG987 (**3**) to increase [^{3}H]NMS dissociation provides an opportunity to assess whether PG987 binds to the same site on the [^{3}H]NMS-occupied receptor as other allosteric agents. The M_3 receptor was used in these experiments because PG987 has the largest speeding effect at this subtype.

The dissociation rate constant (k_{off}) of [^{3}H]NMS was measured at a single time point alone, in the presence of a range of concentrations of PG987, and in the presence of one or more concentrations of a second agent. The data (Fig. 8) are expressed as percentage of control k_{off}, as described under *Materials and Methods*, and fitted to eq. 7.

Strychnine reduced the maximum speeding effect of PG987

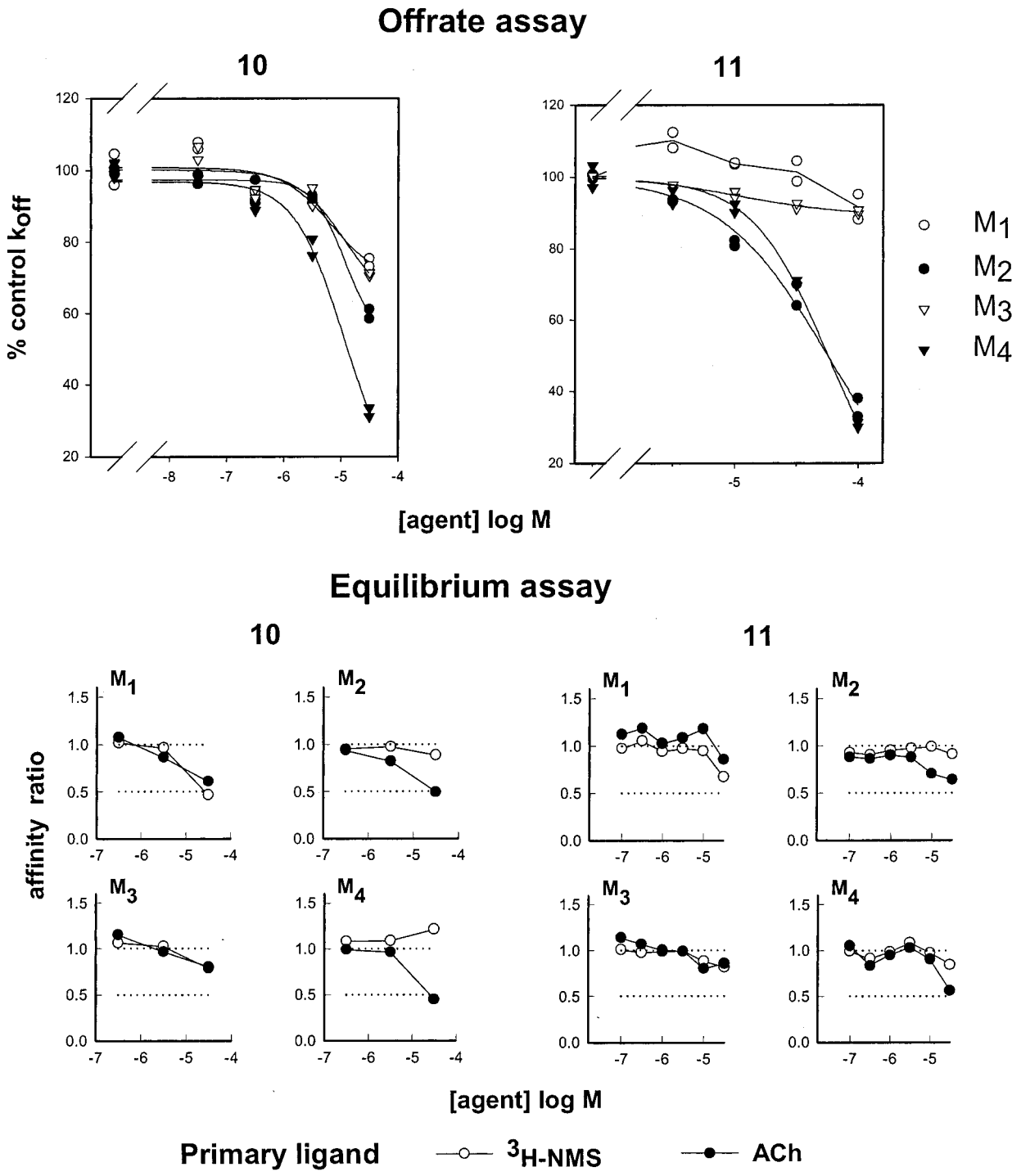

Fig. 5. Effects of steroid compounds **10** and **11** in the off-rate and equilibrium assays. For more information, see the legend to Fig. 2.

on the [^{3}H]NMS dissociation rate constant, E_{max}, but did not affect its EC_{50}. This indicates that it was acting at a site different from that occupied by PG987 and there was no cooperative interaction between these two molecules (i.e., they exhibited neutral cooperativity). Gallamine and PG987 also showed neutral cooperativity. In contrast, KT 5720, staurosporine, and WIN 51,708 did not seem to alter the E_{max} of PG987 but reduced its potency. The data were well fitted assuming a competitive interaction, although strong negative cooperativity cannot be ruled out.

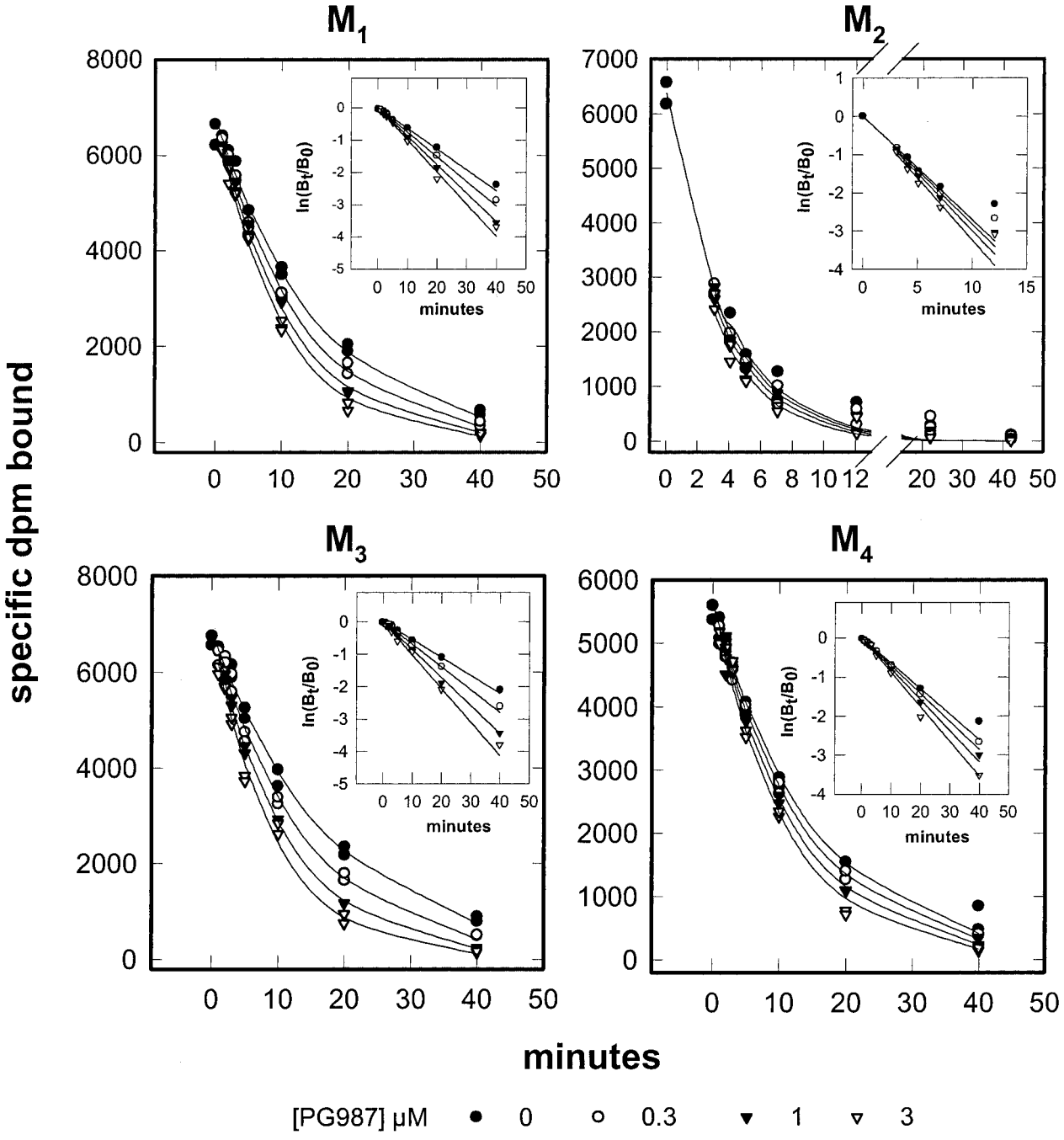

Fig. 6. Dissociation of [^{3}H]NMS from M_1-M_4 receptors, alone and in the presence of three concentrations of PG987. For each receptor subtype, the parameter estimates and standard errors were derived from the nonlinear regression fits of the entire data set to the equation (Lazareno and Birdsall, 1995)

$$B_t = B_0 \times \exp\left(-t \times \frac{X \times K_{occ} \times k_{offX} + k_{off}}{1 + X \times K_{occ}}\right)$$

where t is time, B_0 and B_t are specific binding at dissociation times 0 and t, respectively, X is the concentration of allosteric agent, k_{off} and k_{offX} are the dissociation rate constants of [^{3}H]NMS from the free and PG987-liganded receptor, respectively, and K_{occ} is the affinity of PG987 for the [^{3}H]NMS-occupied receptor. The insets show the linearizing transformation $\ln(B_t/B_0)$ versus time. The parameter estimates (k_{off} min^{-1}, k_{offX} as a percentage of k_{off}, and log K_{occ} log M^{-1}) from the regression analysis at each receptor subtype are: M_1, 0.064, 171, and 6.05; M_2, 0.270, and 132, 5.70; M_3, 0.055, 216, and 5.97; M_4, 0.065, 151, and 5.88.

Discussion

This article describes a new series of compounds that interact allosterically with muscarinic receptors. The initial lead was provided by two commercially available compounds, WIN 51,708 and WIN 62,577. These compounds are potent antagonists at rat NK_1 neurokinin receptors (Venepalli et al., 1992), but the affinity of these compounds, exemplified by WIN 51,708, is 400-fold lower at the human NK_1 receptor (Sachais and Krause, 1994). Our results show, in fact, that WIN 51,708 is up to 60-fold more potent at human muscarinic receptors than at human NK_1 receptors. WIN 62,577 and WIN 51,708 cross the blood-brain barrier (Ukai et al., 1995; Nikolaus et al., 1999; De Araujo et al., 2001), which is a prerequisite for a therapy for human central nervous system disorders.

These two compounds, and the analogs described in this

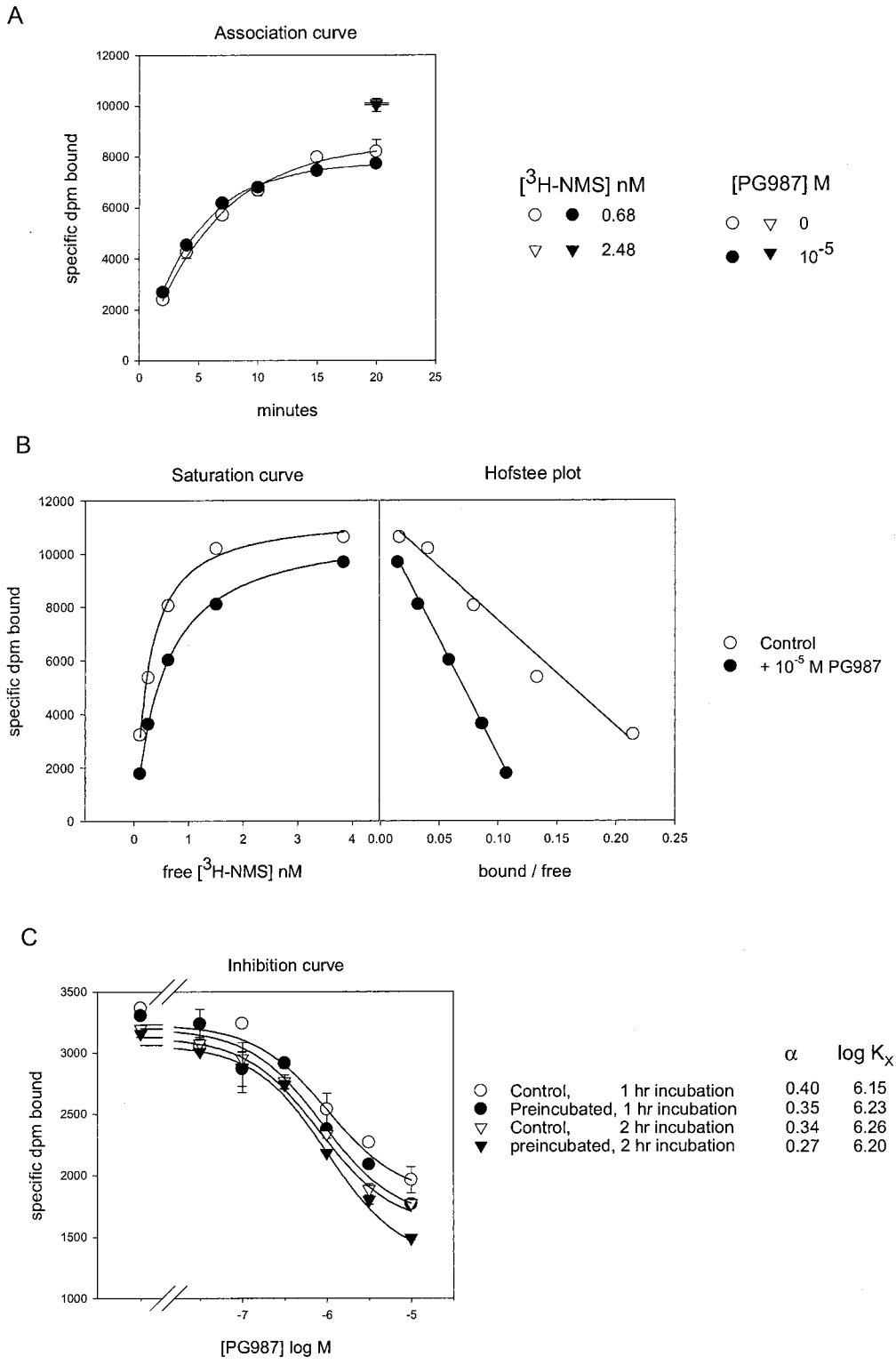

Fig. 7. A, effect of 10 μM PG987 on [^{3}H]NMS association to M_3 receptors. Binding of 0.68 nM [^{3}H]NMS was measured at the indicated incubation times, and binding of 2.48 nM [^{3}H]NMS was measured after 20 min, both in the absence and presence of PG987. The data were fitted to the following equations to yield estimates of k_{on}, K_d and B_{max}: $B_t = B_{eq} \times [1 - \exp(-k_{obs} \times t)]$, $B_{eq} = B_{max} \times [[^3H]NMS]/(K_d + [[^3H]NMS])$, and $k_{obs} = k_{on} \times (K_d + [[^3H]NMS])$, where B_t is specific binding observed after t min and B_{eq} is binding at equilibrium. In this assay, 10 μM PG987 did not affect B_{max}, increased K_d from 0.18 to 0.30 nM (66% increase, affinity ratio of 0.6), and increased k_{on} from 0.190 to 0.222 min^{-1} nM^{-1} (17% increase), leading to a 95% increase in the estimated k_{off}. The assay was repeated twice more with similar results. B, effect of 10 μM PG987 on [^{3}H]NMS K_d and B_{max}. M_3 receptors were incubated with various concentrations of [^{3}H]NMS for 1 h in the absence and presence of PG987. In this assay, PG987 caused 116% increase in K_d and a small (3.7%) but significant reduction in B_{max}. From five assays, 10 μM PG987 increased the K_d of [^{3}H]NMS from 0.19 ± 0.02 to 0.43 ± 0.06 nM, and reduced the B_{max} by 4.9 ± 3.5%. C, reversibility of the effect of PG987. Ten microliters of 10% DMSO (control) or PG987 in 10% DMSO (preincubated) were added to 90 μl of membranes and incubated for 15 min. Then, 900 μl of [^{3}H]NMS was added, followed by 10 μl of PG987 in 10% DMSO (control) or 10 μl of 10% DMSO (preincubated), and the assay was incubated for a further 1 or 2 h. The specific binding data were well fitted to logistic functions with slope fixed at 1, to yield the parameters top, bottom, and IC_{50}. The log affinity of PG987 (log K_X) and cooperativity with [^{3}H]NMS (α) were calculated from the parameters of the fit, the concentration of [^{3}H]NMS (L) and the affinity of [^{3}H]NMS ($K_L = 1/K_d$) with the equations E_{max} = bottom/top, $\alpha = E_{max}/[1 + L \times K_L \times (1 - E_{max})]$, and $K_X = (1 + L \times K_L)/[IC_{50} \times (1 + \alpha \times L \times K_L)]$. The estimates of cooperativity and log affinity from this assay are shown in the figure. From four such assays, preincubation with PG987 did not affect its log affinity (6.04 ± 0.22 control, 6.05 ± 0.07 preincubated) but reduced its cooperativity with [^{3}H]NMS from 0.43 ± 0.07 to 0.30 ± 0.02, a change that just reached statistical significance (p = 0.050, one-tailed paired t test).

article, exhibit positive, neutral, and low negative cooperativity with NMS and especially ACh. This latter characteristic is important in that it suggests the possibility of synthesizing allosteric enhancers that are positively cooperative with ACh at one muscarinic receptor subtype and neutrally cooperative with ACh (and therefore inactive at any concentration) at the other subtypes. This form of selectivity, based on cooperativity rather than affinity, has been termed 'absolute subtype selectivity' (Lazareno et al., 1998; Birdsall et al., 1999) and is a direct consequence of the ternary complex allosteric model (Lazareno and Birdsall, 1995), which underpins the analyses of all our binding and functional data.

Because cooperativity is the ratio of affinities at the liganded and free receptor, it is not surprising that close analogs of an active compound will show quantitative and qualitative changes in cooperativity. In an earlier study (Lazareno et al., 1998), we found that brucine had positive cooperativity with ACh only at the M_1 receptor: *N*-substituted analogs were not positively cooperative with ACh at M_1 receptors but showed positive cooperativity with ACh at other subtypes. In this study, the cooperativity of ACh with the analogs was in general more negative than with the WIN compounds, but the strong negative cooperativity of the WIN compounds with ACh at M_4 receptors became weaker with **3** and **6**. With regard to [^{3}H]NMS, both WIN compounds showed small negative cooperativity at M_1 and M_3 receptors, which became small positivity at both subtypes with **1** and strong positive cooperativity at M_1 receptors with **6**. It is encouraging for potential drug development that analogs with increased affinity (**1**, **3**, and **6**) continue to show favorable cooperativity with ACh (low negative, neutral, or positive cooperativity).

The ternary complex allosteric model implies that the affinity of a compound for the [^{3}H]NMS-occupied receptor can be estimated in two ways: as the product of affinity for the free receptor and cooperativity with [^{3}H]NMS from equilibrium assays and as the reciprocal of the EC_{50} from off-rate

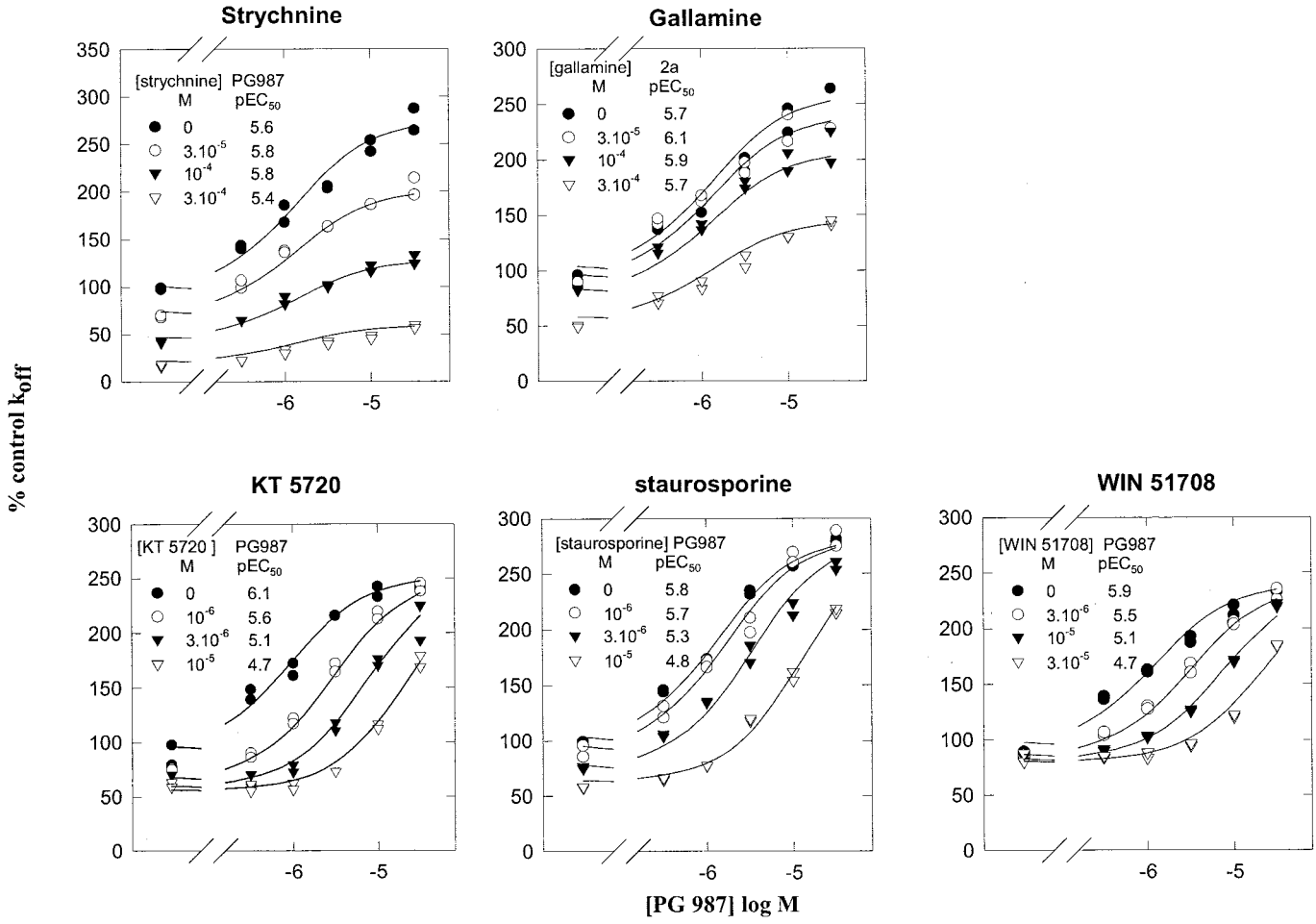

Fig. 8. Concentration-effect curves for PG987 on [^{3}H]NMS dissociation from M_3 receptors, alone and in the presence of three concentrations of test agent, measured at a single time point. The data were converted to dissociation rate constants (see *Materials and Methods*) and expressed as a percentage of the control dissociation rate constant. The curves show the fits from nonlinear regression analysis to eq. 7 under *Materials and Methods*. For strychnine and gallamine, dissociation of [^{3}H]NMS from the dually or triply liganded receptor was not different from 0, and the cooperative interaction with PG987 was not different from 1 (i.e., neutral cooperativity), so these values were fixed. For KT5720, staurosporine, and WIN 51,708, the cooperative interaction with PG987 was not different from 0 (i.e., competition), so this value was fixed. The insets show the $-\log EC_{50}$ values for PG987, obtained from fitting the curves to a logistic function with shared slope factors, and, for KT5720, staurosporine, and WIN 51,708, a shared E_{max} value. Each assay was repeated at least once, with similar results. The estimates (mean ± S.E.) of log affinity and asymptotic [^{3}H]NMS off-rate as a fraction of control (from *n* experiments) were: KT5720, 6.18 ± 0.07 and 0.54 ± 0.02 (3); staurosporine, 5.79 ± 0.18 and 0.57 ± 0.01 (3); WIN 51,708, 5.8 ± 0.1 and 1.0 ± 0.2 (3); strychnine, 4.1 ± 0.0 and 0 (4); gallamine, 3.4 ± 0.0 and 0 (2). From the assays in this figure and replicate assays (n = 13, mean ± S.D.) the dissociation rate constant of [^{3}H]NMS was 0.054 ± 0.011 min^{-1}, the log affinity of PG987 was 5.90 ± 0.15, and the dissociation rate constant of [^{3}H]NMS from the PG987-occupied M_3 receptor as a fraction of control was 2.61 ± 0.33.

assays. Table 1 includes a comparison between the values of log K_{NMSocc} (from equilibrium studies) and $-\log EC_{50}$ (from off-rate studies) for those compounds in which there are at least two observations of each type of measure. Of the 29 comparisons [mean difference = 0.02 ± 0.33 (S.D.)], 24 show a discrepancy between the two measures of less than 3-fold and only one discrepancy is greater than 4-fold. Overall, the data pass this rather stringent test and are therefore consistent with the ternary complex allosteric model as the underlying mechanism responsible for effects on both equilibrium binding of [^{3}H]NMS and ACh and on [^{3}H]NMS dissociation.

WIN 51,708, WIN 62,577, and the analogs examined can be considered in simplistic terms as a fusion of an aromatic heterocyclic system with an alicyclic ring system, especially a steroid structure. Modification of either moiety can generate substantial changes in affinity, cooperativity, and subtype selectivity. Even a subtle change, such as the presence of a double bond in WIN 62,577, has a 10-fold effect on its affinity for the [^{3}H]NMS-occupied M_4 receptor relative to that found for WIN 51,708. The substituents at the 17-position of the steroid ring can also clearly be important; all compounds examined that have a 17-β-hydroxyl group are active and those with a 17-keto function are inactive. Compounds in which the steroid and heterocyclic rings are truncated (e.g., **5** to **8**) are also allosteric.

Most surprisingly, both the steroid moiety alone, for example **10** and **11** (but not some other analogs) and the heterocycle **9**, are individually capable of interacting allosterically with [^{3}H]NMS and with comparable affinities. This result implies that these compounds may interact with different but contiguous or partially overlapping subdomains of the same pharmacophore of an allosteric site. This finding is reminiscent of the simultaneous binding of 2,4-diaminopyrimidine and *p*-aminobenzoyl-glutamate (subcomponent moieties of methotrexate) to dihydrofolate reductase and their allosteric interactions with coenzyme analogs (Birdsall et al., 1978, 1980).

It has generally been assumed that muscarinic ligands that bind to the primary or, especially, allosteric site should have a basic nitrogen, although a few uncharged competitive antagonists ('carbo' analogs) have been described previously (Barlow and Tubby, 1974; Waelbroeck et al., 1996). The allosteric actions of the steroids, **10** and **11**, mean that this requirement is not essential.

In contrast to most muscarinic allosteric agents, many of the compounds investigated in this study do not inhibit the dissociation of [^{3}H]NMS completely at high concentrations. They often only produce a 2-fold or lower slowing effects on the kinetics and, in some instances, especially at M_3 receptors, very small effects indeed (Figs. 2A and 3). In the field of muscarinic receptors, the usual finding is that allosteric ligands completely inhibit the association or dissociation of [^{3}H]NMS from the receptors (Ellis, 1997; Caulfield and Birdsall, 1998; Christopoulos et al., 1998; Holzgrabe and Mohr, 1998), the exceptions being obidoxime (Ellis and Seidenberg, 1992, 2000) and our recent report of the allosteric effects of indolocarbazoles, including staurosporine and KT5720 (Lazareno et al., 2000).

One compound, PG987 (**3**), has the unique effect of increasing the dissociation rate of [^{3}H]NMS; the largest effect is observed at M_3 receptors and the smallest effect at M_2 receptors. This subtype dependence is opposite that found for the other analogs, which slow [^{3}H]NMS dissociation. It is noteworthy that the data from equilibrium and off-rate studies with PG987 are entirely consistent with the allosteric model [i.e., there are no discrepancies between estimates of affinity for the [^{3}H]NMS-occupied receptor from the two types of assay, and the slope factors were 1 or close to 1 (Table 1)]. The agreement occurs despite the fact that enhancement of [^{3}H]NMS dissociation by PG987 is opposite the inhibitory effect seen with all the other compounds in this study, and in every other published study of allosteric agents at muscarinic receptors. Different effects of PG987 might be seen with other radioligands and incubation conditions: for example, gallamine enhances the dissociation rate of the antagonist [^{3}H]QNB (but not [^{3}H]NMS) in conditions of low (but not high) ionic strength (Ellis and Seidenberg, 1992, 2000). Allosteric ligands can also enhance the dissociation rate of antagonists from other G protein-coupled receptors, for example α_{1A} and α_{2A} adrenoceptors (Leppik et al., 1998, 2000).

This unique effect of PG987 allowed us to assess whether other allosteric agents inhibit [^{3}H]NMS dissociation by acting at the same site as PG987. M_3 receptors were used because PG987 had the largest effect at this subtype. Figure 8 shows the effect of five compounds on concentration-effect curves of PG987 speeding [^{3}H]NMS dissociation. Strychnine and gallamine did not affect the EC_{50} of PG987 but progressively reduced the E_{max}. The data were fitted to a model in which the [^{3}H]NMS-occupied receptor contains two distinct allosteric sites (see *Materials and Methods*). The data fitted the model well, with the cooperativity between the two sites not different from 1, assuming that [^{3}H]NMS cannot dissociate from receptors with strychnine or gallamine bound, both singly and simultaneously with PG987. This result suggests that PG987 and gallamine or strychnine can simultaneously occupy distinct allosteric sites on the [^{3}H]NMS-occupied M_3 receptor and show neutral cooperativity with each other.

In contrast, KT 5720, staurosporine, and WIN 51,708 seem to bind to the same site as PG987 on the [^{3}H]NMS-liganded M_3 receptor. These agents caused a concentration-dependent reduction in the potency of PG987 but no change in the slope or E_{max} (Fig. 8). The data fitted the model well with cooperativity not different from zero (i.e., the compounds behaved competitively), although strong negative cooperativity cannot be ruled out.

It is worth noting that WIN 51,708 alone has *no effect* on [^{3}H]NMS dissociation from M_3 receptors, although it clearly occupies the receptors and inhibits the effects of PG987: it acts as a 'neutral antagonist' of PG987 in this kinetic assay. Obidoxime acts in a similar manner at the 'common allosteric site' (i.e., the site that binds gallamine), in that it has no effect on the dissociation of [^{3}H]QNB from the M_2 receptor (at low ionic strengths) but competes with other ligands that do affect [^{3}H]QNB dissociation (Ellis and Seidenberg, 2000).

In conclusion, members of this new series of muscarinic allosteric agents are relatively potent and, like brucine, can support positive cooperativity with ACh. They do not, however, bind to the same allosteric site on M_3 receptors as gallamine and strychnine (and probably brucine) but may bind to the same allosteric site as KT5720 and staurosporine. They can enhance, inhibit, or have no effect on the dissociation rate of [^{3}H]NMS. Two compounds, PG987 and WIN 51,708, can be used to provide a test of whether another

allosteric agent binds to the 'WIN' allosteric site on the M_3 muscarinic receptor.

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