0026-895X/06/7002-656–666$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:656–666, 2006

Vol. 70, No. 2
23762/3126229
Printed in U.S.A.

Differences in Kinetics of Xanomeline Binding and Selectivity of Activation of G Proteins at M_1 and M_2 Muscarinic Acetylcholine Receptors

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Received February 23, 2006; accepted May 4, 2006

ABSTRACT

Xanomeline is a functionally selective M_1/M_4 muscarinic acetylcholine receptor agonist that nevertheless binds with high affinity to all five subtypes of muscarinic receptors. A novel mode of interaction of this ligand with the muscarinic M_1 receptors characterized by persistent binding and receptor activation after extensive washout has been shown previously. In the present study, using human M_1 and M_2 receptors expressed in Chinese hamster ovary cells and [^{3}H]*N*-methylscopolamine as a tracer, we show that persistent binding of xanomeline also occurs at the M_2 receptor with similar affinity as at the M_1 receptor (K_I = 294 and 296 nM, respectively). However, kinetics of formation of xanomeline wash-resistant binding to M_2 receptors was markedly slower than to M_1 receptors. Xanomeline was a potent fast-acting full agonist in stimulating guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding at M_1 receptors, whereas at M_2 receptors it behaved as a potent partial agonist (40% of carbachol maximal response) only upon preincubation for 1 h. Development of xanomeline agonistic effects at the M_2 receptor was slower than its ability to attenuate carbachol responses. We also demonstrate that xanomeline discriminates better between G protein subtypes at M_1 than at M_2 receptors. Our data support the notion that xanomeline interacts with multiple sites on the muscarinic receptor, resulting in divergent conformations that exhibit differential effects on ligand binding and receptor activation. These conformations are both time- and concentration-dependent and vary between the M_1 and the M_2 receptor.

Muscarinic acetylcholine receptors mediate a wide variety of physiological functions (Caulfield, 1993). Five subtypes of muscarinic acetylcholine receptors have been cloned (Bonner et al., 1987), and each is involved in mediating specific functions. For this reason, subtype-selective muscarinic ligands with potential therapeutic use have been pursued for several decades. For example, M_1 receptors take part in cognitive processes and formation of memory. Many studies have documented that in the course of natural aging and particularly in Alzheimer's disease, there is marked loss of cholinergic neurons in basal forebrain and their terminals in the brain cortex and hippocampus (Perry et al., 1977a,b; Bartus et al., 1982; Francis et al., 1999; Dolezal and Kasparova, 2003). This decrease in cholinergic input is not accompanied by changes in the density of postsynaptic M_1 receptors (Ladner and Lee, 1998). Therefore, an agonist that works selectively at the M_1 muscarinic receptor might improve memory in patients with Alzheimer's disease without eliciting serious side effects mediated by other muscarinic receptor subtypes. However, the high homology among subtypes of muscarinic receptors in the transmembrane domain where the acetylcholine binding site is located makes the search for selective ligands difficult, with more success in discovering receptor antagonist than agonist ligands. To date, only a few selective agonists have been described. One of them is xanomeline (3-[3-hexyloxy-1,2,5-thiadiazo-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine), which has been identified as a functionally selective potent agonist for M_1 and M_4 receptors (Shannon et al., 1994; Ward et al., 1995; Bymaster et al., 1997, 1998). Strikingly, despite its functional selectivity, no major differences in the affinity of xanomeline binding to individual subtypes of muscarinic receptors have been found (Bymaster et al., 1997; Watson et al., 1998; Wood et al., 1999). The mechanism of functional selectivity of xanomeline therefore remains unknown.

A remarkable feature of xanomeline action is its ability to

This study was financed by Research Project AV0Z 5011922 supported by Grant Agency of the Czech Republic grant 305/05/0452 and Research Center grant LC554, and by National Institutes of Health grant NS25743.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.023762.

ABBREVIATIONS: NMS, *N*-methylscopolamine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary; ANOVA, analysis of variance.

stimulate M_1 muscarinic receptors even after intensive washing to remove the free ligand (Christopoulos et al., 1998, 1999). It has been demonstrated that xanomeline binds to M_1 muscarinic receptors in two ways: reversibly to the orthosteric binding site where conventional muscarinic agonists and competitive antagonists bind and firmly to another site that is close to but not identical to the orthosteric binding site. Binding of xanomeline to this ectopic site is resistant to washing and is accompanied by persistent receptor activation. It also modulates binding of ligands to the orthosteric site of the receptor in a complex manner (Jakubík et al., 2002). However, wash-resistant binding of xanomeline as such cannot explain its functional selectivity because it is not confined to the M_1 receptor subtype. A similar mode of xanomeline binding to the M_5 receptor, for example, has recently been shown (Grant and El-Fakahany, 2005). However, xanomeline avid binding in this case results in persistent antagonism of receptor activation by agonists.

The aim of our experiments was to get further insight into the basis of xanomeline mechanisms of action and functional selectivity. To this end, we compared the mechanism of xanomeline binding to and activation of a pair of muscarinic receptors where xanomeline exhibits marked differential efficacy. We chose the M_1 receptor, where xanomeline behaves as a potent and efficacious agonist, and the M_2 receptor, where xanomeline binds equally well but does not result in receptor activation. M_1 receptors preferentially couple to the G_q/G_{11} family of heterotrimeric G proteins that lead to activation of phospholipase C, whereas M_2 receptors preferentially couple to the G_i/G_o family of G proteins that result in inhibition of adenylyl cyclase (Caulfield, 1993). However, besides these principal G proteins, muscarinic receptors also couple to some extent to other G protein classes (Jakubík et al., 1996; Michal et al., 2001; Tucek et al., 2002). Therefore, we examined xanomeline-induced receptor coupling to individual classes of G proteins using the scintillation proximity assay (DeLapp et al., 1999).

In this work, we demonstrate that wash-resistant binding of xanomeline occurs at both the M_1 and M_2 subtypes of muscarinic receptors. However, there are marked differences in the kinetics of formation of wash-resistant xanomeline-receptor complex between these two receptor subtypes. There are also marked differences in the kinetics, potency, and efficacy of activation of various G proteins by xanomeline at these receptors. These differences may contribute to the functional selectivity of this unique muscarinic receptor agonist.

Materials and Methods

Materials. The radioligands [^{3}H]*N*-methylscopolamine chloride ([^{3}H]NMS) and guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) and anti-rabbit IgG-coated scintillation proximity beads were from Amersham (Little Chalfont, Buckinghamshire, UK). Carbachol, dithiothreitol, GDP, GTPγS, and NMS were from Sigma-Aldrich (St. Louis, MO). Xanomeline was kindly provided by Dr. Bymaster (Eli Lilly Research Laboratories, Indianapolis, IN).

Cell Culture and Membrane Preparation. Chinese hamster ovary cells stably transfected with the human M_1 or M_2 muscarinic receptor genes were grown to confluence in 50-cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and were subcultured to 16 100-mm Petri dishes (approximately 2×10^6 cells/dish). Cells were detached by mild trypsinization on day 5 after subculture. Detached cells were washed twice in phosphate-buffered saline and 3-min centrifugation at 250*g*. Washed cells were diluted in ice-cold homogenization medium (100 mM NaCl, 20 mM Na-HEPES, and 10 mM EDTA, pH 7.4) and homogenized on ice by two 30-s strokes using a homogenizer (Ultra-Turrax; Janke and Kunkel GmbH and Co. KG, IKA-Labortechnik, Staufen, Germany) with a 30-s pause between strokes. Cell homogenate was centrifuged for 30 min at 30,000*g*. The supernatant was discarded, and pellets were resuspended in incubation medium (100 mM NaCl, 10 mM $MgCl_2$, and 20 mM Na-HEPES, pH 7.4) and centrifuged for 30 min at 30,000*g*. Pellets were kept at −20°C until assayed for 10 weeks at maximum.

Treatment with Xanomeline. Two types of experiments with xanomeline were carried out. In experiments referred to as "continuous presence", xanomeline was present during incubation with radioligands. In experiments referred to as "prelabeling/washing" to determine xanomeline "wash-resistant binding", membranes were preincubated for 60 min at 30°C with the indicated concentrations of xanomeline, centrifuged for 30 min at 30,000*g* at 4°C, and resuspended in incubation medium. Centrifugation and resuspension were repeated three times with a 30-min waiting period in between to ensure removal of free xanomeline in the medium. Alternatively, xanomeline was added to intact cells in case of measurement of kinetics of its binding or activation of [^{35}S]GTPγS (Figs. 2 and 4) to expedite first steps of washing. Cells were treated with xanomeline at 30°C for the indicated times and then centrifuged for 1 min at 300*g*. The medium was quickly removed then cells were resuspended in incubation medium and immediately recentrifuged. Washed cells were disrupted by hypo-osmotic shock and rapid freezing followed by thawing, followed by the addition of 10 mM EDTA and membrane preparation as described above.

Radioligand Binding Experiments. All radioligand binding experiments were carried out in 96-well plates at 30°C in the incubation medium described above supplemented with freshly prepared dithiothreitol at a final concentration of 1 mM. Incubation volume was 200 μl. Binding of xanomeline to muscarinic receptors was determined by its ability to decrease binding of 1 nM [^{3}H]NMS. Nonspecific binding was determined in the presence of 10 μM NMS. Incubation with [^{3}H]NMS lasted for 60 min and was terminated by filtration on glass fiber filters. For determination of [^{35}S]GTPγS binding to G proteins in membranes, a final concentration of 200 pM (M_1 receptors) or 500 pM (M_2 receptors) [^{35}S]GTPγS was used, supplemented by 5 μM (M_1 receptors) or 50 μM (M_2 receptors) GDP. [^{35}S]GTPγS nonspecific binding was determined in the presence of 1 μM GTPγS. Incubation with [^{35}S]GTPγS was carried for 20 min, and free ligand was removed by filtration through GF/F glass fiber filters (Whatman, Clifton, NJ) using a Mach III cell harvester (Tomtec, Hamden, CA). Filters were dried in a vacuum for 1 h while being heated at 80°C, and then solid scintillator Meltilex A was melted on filters (105°C; 90 s) using a hot plate. After cooling, the filters were counted using a Microbeta scintillation counter (PerkinElmer Wallac, Gaithersburg, MD).

Scintillation Proximity Assay. In the scintillation proximity assay, incubation with [^{35}S]GTPγS was terminated by membrane solubilization by the addition of 20 μl of 10% Nonidet P-40. After 20 min, 10 μl of individual primary antibodies against various G protein α subunits was added, and incubation was continued for 1 h. The final dilution was 1:1000 in case of anti-G_i-α and anti-G_s-α antibodies and 1:2000 in case of the anti-G_q-α antibody. One batch of anti-rabbit IgG-coated scintillation beads was diluted in 40 ml of incubation medium, and 50 μl of the suspension was added to each well for 3 h. Then, plates were centrifuged for 15 min at 1000*g* and counted using the scintillation proximity assay protocol in a Microbeta scintillation counter.

Data Analysis. Data were preprocessed by Open Office 1.1.5 (http://www.openoffice.org) and subsequently analyzed by Grace 5.1.18 (http://plasma-gate.weizmann.ac.il/Grace/) and statistic package R (http://www.r-project.org) running on the Mandriva distribution of Linux.

The following equations were fitted to data.

Interference with [^{3}H]NMS binding was calculated by

$$y = 100 \times [1 - x/(IC_{50}{}^{n_H} + x)] \quad (1)$$

where y is binding of [^{3}H]NMS at a concentration of displacer x normalized to binding in the absence of displacer, EC_{50} is the concentration causing 50% decrease in binding, and n_H is the Hill coefficient. Equilibrium dissociation constant of displacer (K_I) was calculated according to Cheng and Prusoff (1973).

Time-dependent decrease in [^{3}H]NMS binding by xanomeline was computed using

$$y = (100 - \text{Plateau}) \times e^{(-K_{obs} \times x)} + \text{Plateau} \quad (2)$$

where y is binding of [^{3}H]NMS at time x normalized to its binding at time 0, plateau, is normalized[^{3}H]NMS binding at steady state, and K_{obs} is observed rate of xanomeline binding.

Time-dependent increase in [^{35}S]GTPγS binding was determined with the equation

$$y = Y_0 + Y_{max} \times [1 - e^{(-K \times x)}] \quad (3)$$

where y is binding of [^{35}S]GTPγS at time x; Y_0 is binding at time 0, Y_{max} is maximum binding, and K is rate constant.

Concentration-response enhancement of [^{35}S]GTPγS binding by agonists was calculated as

$$y = 100 + E_{max}/[1 + (EC_{50} - x)^{n_H}] \quad (4)$$

where y is [^{35}S]GTPγS binding in the presence of agonist at concentration x normalized to binding in the absence of agonist, E_{max} is maximal percentage of increase by agonist, EC_{50} is concentration of agonist producing 50% of maximal effect, and n_H is the Hill coefficient.

Analysis of the effects of xanomeline on the concentration-response curves to carbachol using Clark plots (Lew and Angus, 1996) was performed according to the equation

$$pEC_{50} = -\log([x] + 10^{-pK_B}) - \log c \quad (5)$$

where pEC_{50} is the negative logarithm of agonist EC_{50} in the presence of competitor at concentration x, pK_B is the negative logarithm of equilibrium dissociation constant of competitor, and c is fitting constant.

Results

Xanomeline Reversible and Wash-Resistant Binding to M_1 and M_2 Muscarinic Receptors. Experiments were performed on membranes from CHO cells stably expressing M_1 and M_2 receptors (1.8 ± 0.1 and 1.2 ± 0.1 pmol of [^{3}H]NMS binding sites per milligram of protein in membranes, respectively). The equilibrium dissociation constant (K_d) of [^{3}H]NMS to CHO-M_1 and CHO-M_2 membranes was 418 ± 20 and 524 ± 25 pM, respectively, and was the same under all experimental setups. Affinity of interaction of xanomeline with M_1 and M_2 muscarinic receptors was determined by its ability to displace binding of 1 nM [^{3}H]NMS to membranes of CHO cells that stably express each receptor subtype (Fig. 1). The K_I value of xanomeline at the M_1 and M_2 muscarinic receptors was 13.5 ± 1.5 and 37.2 ± 4.1 nM, respectively. Membranes pretreated with xanomeline for 60 min followed by extensive washing exhibited concentration-dependent reduction in subsequent binding of [^{3}H]NMS in the absence of free xanomeline, albeit with lower potency of xanomeline in comparison with that obtained in its continuous presence in the binding assay medium (Fig. 1). The K_I of xanomeline wash-resistant binding to M_1 and M_2 muscarinic receptors was 296 ± 31 and 294 ± 34 nM, respectively. These results thus indicate slightly higher affinity of reversible xanomeline binding to M_1 than M_2 receptors, whereas affinity of its wash-resistant binding is the same for both subtypes.

Kinetics of Xanomeline Wash-Resistant Binding to M_1 and M_2 Muscarinic Receptors. To detect possible differences between M_1 and M_2 receptors in the rate of formation of xanomeline wash-resistant binding, cells were exposed to xanomeline for various periods and then washed and incubated with [^{3}H]NMS. Decrease in [^{3}H]NMS binding was taken as measure of xanomeline wash-resistant binding. Formation of xanomeline wash-resistant binding at M_1 receptors was extremely fast and already occurred upon washing cells immediately after addition of xanomeline (Fig. 2, left, time 0). The K_{obs} values of this interaction did not follow the concentration dependence expected for a simple bimolecular reaction (Fig. 3). Thus, K_{obs} only doubled by increasing the concentration of xanomeline from 0.3 to 10 μM. Furthermore, the relationship between K_{obs} and xanomeline concentration during preincubation demonstrated saturability (Fig. 3). These findings are similar to those we reported previously (Jakubík et al., 2002). In contrast, formation of xanomeline wash-resistant binding at M_2 receptors was markedly slower than at M_1 receptors (Fig. 2, right). Most notably, there was no evidence of the instantaneous phase of xanomeline wash-resistant binding shown with M_1 receptors. Moreover, increasing the concentration of xanomeline during preincubation resulted in more marked increase in K_{obs} values of its wash-resistant binding at the M_2 receptor. For example, increasing xanomeline concentration from 0.3 to 10 μM changed K_{obs} by almost 10-fold. However, the relationship between xanomeline concentration and K_{obs} at the M_2 receptor still deviated from a simple bimolecular scheme of interaction (Fig. 3), even though the deviation was less marked than in the M_1 receptor.

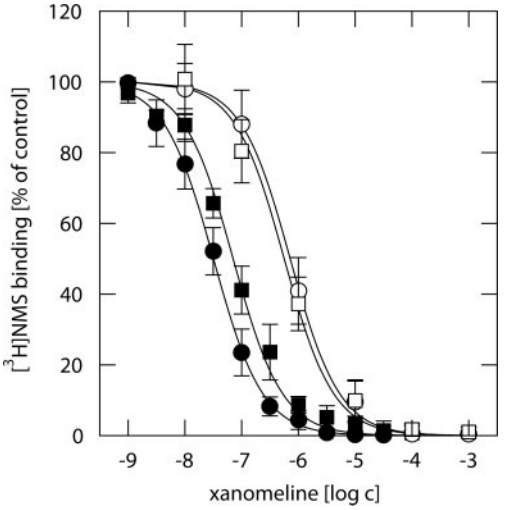

Fig. 1. Lack of selectivity of xanomeline binding at M_1 and M_2 muscarinic receptors. [^{3}H]NMS binding to membranes from CHO cells expressing M_1 (circles) or M_2 (squares) subtypes of muscarinic receptor was determined in the continuous presence of increasing concentrations of xanomeline (closed symbols). Alternatively, membranes were preincubated with increasing concentrations of xanomeline for 60 min, washed, and then incubated with [^{3}H]NMS (open symbols). Data are means ± S.E.M. of three to four independent experiments performed in triplicate. Hill slopes are not significantly different from unity ($P < 0.05$; Wilcoxon test).

Kinetics of Xanomeline Wash-Resistant Activation of M_1 and M_2 Receptors. In related experiments, we explored possible differences in the rate of xanomeline wash-resistant activation of M_1 and M_2 receptors. Membranes were exposed to xanomeline for various periods and then washed and incubated with [^{35}S]GTPγS. Increase in [^{35}S]GTPγS binding was taken as a measure of receptor activation by persistently bound xanomeline. Preincubation with 10 μM xanomeline followed by washing resulted in a time-dependent activation of [^{35}S]GTPγS binding at M_1 and M_2 receptors, with a higher response at the former receptor (Fig. 4). The stimulatory effects of wash-resistant xanomeline at both subtypes were abolished by NMS. Similar to differences in the kinetics of wash-resistant binding of xanomeline at M_1 and M_2 receptors, xanomeline exhibited slower wash-resistant activation of [^{35}S]GTPγS binding at the M_2 receptor. Appearance of xanomeline wash-resistant activation of muscarinic receptors was significantly slower than the formation of xanomeline wash-resistant inhibition of [^{3}H]NMS binding ($P < 0.05$; unpaired t test). For example, although preincubation with 10 μM xanomeline resulted in an instantaneous 35% wash-resistant decrease in [^{3}H]NMS binding at M_1 receptors (Fig. 2), there was no corresponding enhancement of [^{35}S]GTPγS binding (Fig. 4). Furthermore, although wash-resistant binding of 10 μM xanomeline to the M_1 receptor reached equilibrium at 10 min, [^{35}S]GTPγS binding continued to increase between 10 and 30 min of preincubation with xanomeline. In more quantitative terms, the rates of formation of xanomeline wash-resistant binding to the M_2 receptor and persistent activation of [^{35}S]GTPγS binding were 0.318 ± 0.021 and 0.0795 ± 0.0032 min^{-1}, respectively.

Interactions of Xanomeline and Carbachol in Receptor Activation. Agonistic properties of xanomeline and its interaction with carbachol were measured as stimulation of [^{35}S]GTPγS binding to membranes. Because of the complex nature of xanomeline binding, it was necessary to use different experimental setups: 1) simultaneous addition of xanomeline or carbachol or their combination and [^{35}S]GTPγS to measure immediate effects of xanomeline; 2) preincubation of membranes with xanomeline for 60 min preceding incubation with [^{35}S]GTPγS, with or without carbachol. In this setup, both immediate as well as delayed effects of xanomeline are measured; and 3) preincubation with xanomeline for 60 min followed by washing and incubation with [^{35}S]GTPγS in the absence or in the presence of carbachol. In this protocol, only effects of xanomeline wash-resistant binding are measured.

Measurements of concentration-response curves of xanomeline, carbachol, and carbachol in the presence of xanomeline in stimulating [^{35}S]GTPγS binding are shown in Fig. 5. Curve parameters obtained by fitting eq. 4 to data from individual experiments are summarized in Table 1. In concert with reported functional selectivity of xanomeline, simultaneous addition of xanomeline and [^{35}S]GTPγS stimulated [^{35}S]GTPγS binding at M_1 but not at M_2 receptors. In contrast, carbachol stimulated [^{35}S]GTPγS binding at both subtypes with the same potency and efficacy (Fig. 5, top; Table 1). At M_1 receptors, xanomeline demonstrated slightly but significantly higher efficacy and more than 100 times higher potency than carbachol ($P < 0.05$; unpaired t test). The potency of carbachol in stimulating [^{35}S]GTPγS binding gradually decreased in the presence of increasing concentrations of xanomeline without a change in carbachol efficacy.

At the M_1 receptor, preincubation in the presence of agonists for 60 min before the addition of [^{35}S]GTPγS resulted in similar effects of xanomeline, carbachol, and their combination on [^{35}S]GTPγS binding compared with simultaneous addition of the agonists with [^{35}S]GTPγS (Fig. 5, middle). At the M_2 receptor, however, a small but significant stimulatory effect of xanomeline was observed (pEC_{50} = 7.78 ± 0.03; E_{MAX} = 1.75 ± 0.05-fold over basal), in contrast to the lack of agonistic activity upon simultaneous addition of xanomeline and [^{35}S]GTPγS (Fig. 5, middle; Table 1) ($P < 0.05$, ANOVA followed by Dunnett's post test). However, there was no difference in the antagonistic effects of xanomeline on carbachol in the two experimental protocols (Table 2).

Preincubation with xanomeline for 60 min followed by extensive washing resulted in concentration-dependent enhancement of [^{35}S]GTPγS binding at both M_1 or M_2 receptors (Fig. 5). Xanomeline wash-resistant receptor activation exhibited the same efficacy at both receptors as in preincubation with xanomeline before addition of [^{35}S]GTPγS but without washing away free xanomeline. However, the potency of xanomeline was significantly lower in the washout protocol, being reduced by 143- and 93-fold at the M_1 and the M_2 receptor, respectively ($P < 0.05$; unpaired t test). Moreover, wash-resistant binding of low concentrations of xanomeline did not shift the concentration-response curve to carbachol at M_1 or M_2 receptors in spite of causing wash-resistant receptor activation (Fig. 5, bottom; Table 1). Thus, the potency of

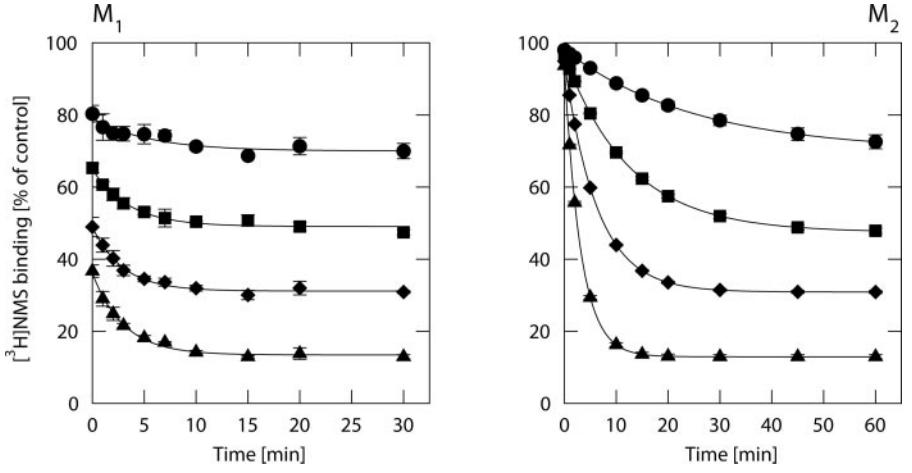

Fig. 2. Time course of formation of xanomeline wash-resistant binding to M_1 and M_2 muscarinic receptors. Intact CHO cells expressing M_1 or M_2 receptors were pretreated for the indicated times with increasing concentrations of xanomeline followed by washing and membrane preparation and determination of [^{3}H]NMS binding to M_1 (left) and M_2 (right) receptors. ●, 0.3 μM; ■, 1 μM; ♦, 3 μM; and ▲, 10 μM xanomeline. Data are means ± S.E.M. of three independent experiments performed in quadruplicate.

persistently bound xanomeline is higher at causing receptor activation and at decreasing [^{3}H]NMS binding to the receptor than at antagonism of receptor activation by carbachol. In addition, in the preincubation/washing procedure xanomeline (3 and 10 μM) decreased the maximal response to carbachol at M_2 but not at M_1 receptors (Table 1).

Analysis of the interaction between xanomeline and carbachol by the method of Kaumann and Marano (1982) that compares carbachol concentrations required to produce equal responses in the absence and in the presence of xanomeline (i.e., equal fractional receptor occupancy) yielded slopes significantly different from unity in all cases ($P < 0.05$; Wilcoxon test). In the continuous presence of xanomeline, the slopes are greater than 1 at both receptor subtypes, suggesting possible interaction with more than one molecule of xanomeline with the receptor. In contrast, slopes are smaller than 1 in the preincubation/washing procedure at both subtypes, indicating deviation from a competitive interaction. Therefore, this method is not suitable for estimation of the equilibrium dissociation constant of xanomeline-receptor interaction.

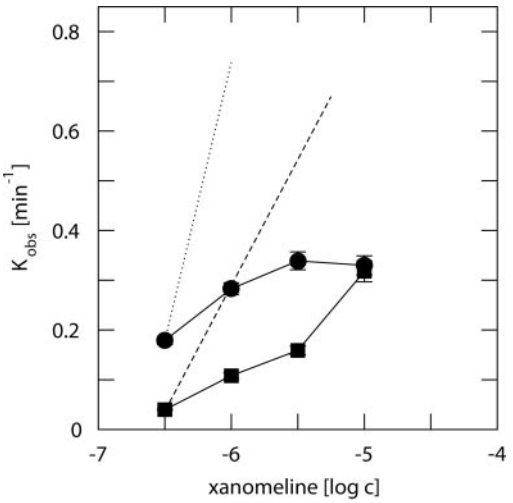

Fig. 3. Comparison of observed rate of loss of [^{3}H]NMS binding at M_1 and M_2 muscarinic receptors by xanomeline preincubation. Observed rate constants K_{obs} of loss [^{3}H]NMS to M_1 (●) or M_2 (■) receptors obtained by fitting eq. 2 to data in Fig. 2 are plotted against the concentration of xanomeline present during pretreatment. Means ± S.E.M. of fits from individual experiments are displayed and connected with solid lines. Dotted (M_1) and dashed (M_2) lines represent theoretical values of K_{obs} based on extrapolation of K_{obs} measured at 300 nM xanomeline and assuming a simple bimolecular reaction of xanomeline with the receptor.

Effects of xanomeline on carbachol concentration-response curves were therefore analyzed by Clark's nonlinear regression as described by Lew and Angus (1996). In this analysis, pEC_{50} values obtained by fitting eq. 4 to carbachol concentration-response curves in the presence of xanomeline (Fig. 5, closed symbols; Table 1) were plotted against the logarithm of xanomeline concentration (Fig. 6), and eq. 5 was fitted to the data. This analysis was applied to the data from individual experiments, and means ± S.E.M. are shown in Table 2. Estimates of the xanomeline equilibrium dissociation constant (K_B) in its continuous presence at both receptor subtypes are equal to the corresponding xanomeline concentration that produces half-maximal receptor activation (EC_{50}). However, for the preincubation/washing procedure the estimated xanomeline K_B as an antagonist is lower than its EC_{50} values as an agonist at both M_1 and M_2 receptors. However, although at M_1 receptors K_B of xanomeline is only 2 times higher than its EC_{50}, the corresponding ratio is more than 10-fold at the M_2 receptor. Preincubation with xanomeline caused lowering of its K_B at the M_2 receptor by 2.5-fold (enhanced affinity), but it did not alter the corresponding value at the M_1 receptor. Washing xanomeline out after preincubation resulted in marked increases in K_B values. Thus, washing reduced the potency of xanomeline in antagonizing the effects of carbachol by 300- and 1000-fold at M_1 and M_2 receptors, respectively.

Activation of Various G Proteins by Xanomeline and Carbachol at M_1 and M_2 Receptors. Selectivity of xanomeline and carbachol in activating receptor coupling with various subtypes of G proteins was studied using scintillation proximity assays. Figure 7 and Table 3 show stimulation of [^{35}S]GTPγS binding by carbachol or xanomeline to the G_i, G_s, and G_q subtypes of G proteins at M_1 or M_2 receptors. At M_1 receptors, simultaneous addition of either carbachol or xanomeline with [^{35}S]GTPγS preferentially activated G_q with equal high efficacy. Both agonists also activated G_i and G_s but with lower efficacy and potency. Xanomeline exhibited more selectivity than carbachol in activating various subtypes of G proteins, in terms of differential higher potency and efficacy at G_q on the one hand and G_s and G_i on the other hand. A similar pattern was observed after 60-min preincubation with agonists before the addition of [^{35}S]GTPγS.

Efficacy of xanomeline in stimulating G_i [^{35}S]GTPγS bind-

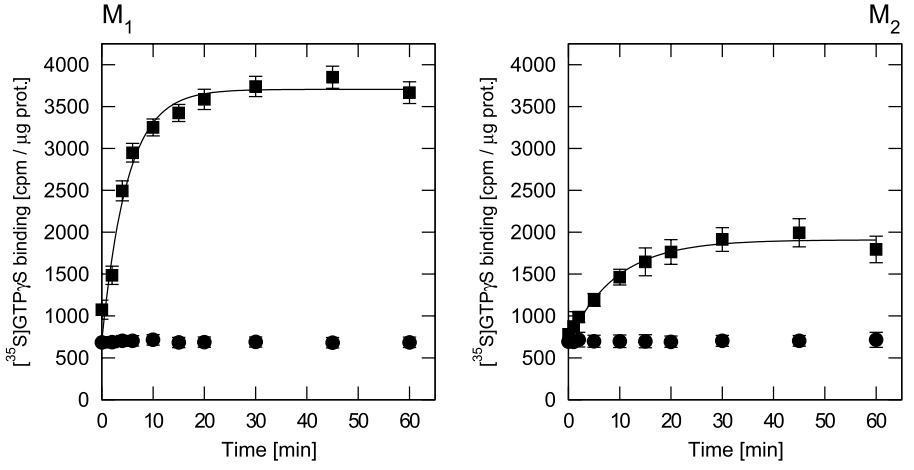

Fig. 4. Time course of xanomeline wash-resistant receptor activation. CHO cells expressing M_1 (left) or M_2 (right) receptors were pretreated with 10 μM xanomeline for the indicated times followed by washing, membrane preparation, and determination of [^{35}S]GTPγS binding. ■, 10 μM xanomeline alone; and ●, 10 μM xanomeline in the presence of 10 μM NMS. Data are means ± S.E.M. of three independent experiments performed in quadruplicate. Observed rate constants are 0.202 ± 0.013 and 0.0795 ± 0.0032 min^{-1} at M_1 and M_2 receptors, respectively.

ing at M_2 receptors was only 23% that of carbachol when added simultaneously with [^{35}S]GTPγS with very small or no stimulation of G_s or G_q (Fig. 7, top right; Table 3). Effects of carbachol at all three subtypes of G proteins did not change when it was added 60 min ahead of [^{35}S]GTPγS. However, similar preincubation with xanomeline potentiated its ability to stimulate [^{35}S]GTPγS binding at G_i and also uncovered activation of G_s and G_q that was absent when xanomeline

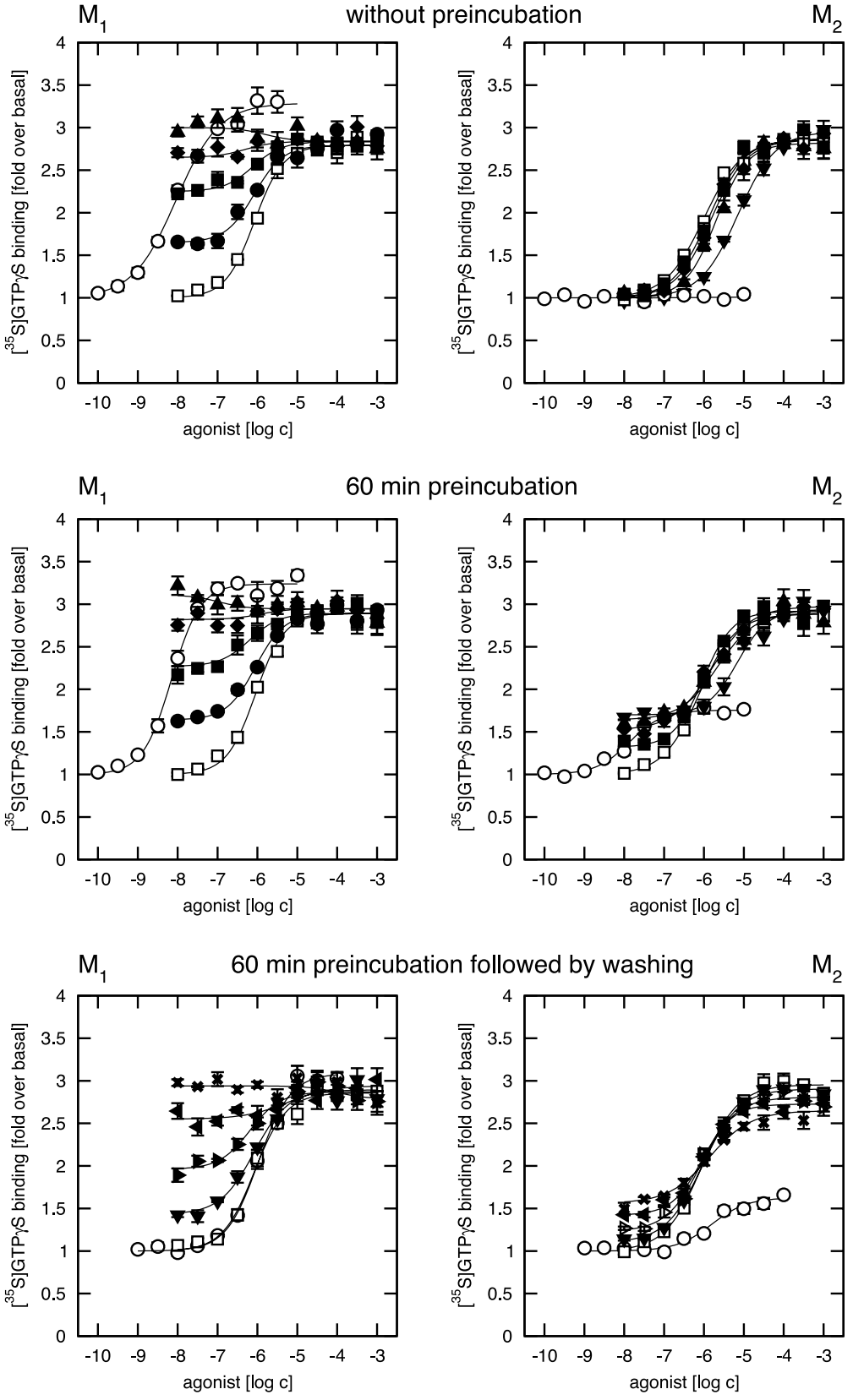

Fig. 5. Stimulation of [^{35}S]GTPγS binding by carbachol and xanomeline. Concentration-response of [^{35}S]GTPγS binding to CHO membranes expressing M_1 (left) or M_2 (right) receptors induced by xanomeline alone (○), carbachol alone (□), or carbachol in the continuous presence of increasing concentrations of xanomeline (●, 3 nM; ■, 10 nM; ♦, 30 nM; ▲, 100 nM; and ▼, 300 nM) or in the preincubation/washing protocol (▼, 300 nM; ▶, 1 μM; ◀, 3 μM; ✚, 10 μM). Top row, agonists were added simultaneously with [^{35}S]GTPγS. Middle row, agonists were added 60 min ahead of [^{35}S]GTPγS. Bottom row, membranes were preincubated for 60 min with increasing concentrations of xanomeline followed by washing and co-addition of [^{35}S]GTPγS with buffer or increasing concentrations of carbachol. Data are means ± S.E.M of four to six independent experiments performed in quadruplicate.

was added simultaneously with the radionucleotide (Fig. 7; Table 3).

Ratios of xanomeline to carbachol potencies also differed between M_1 and M_2 receptors. Although xanomeline present during 60-min preincubation was more than 140 times more potent than carbachol at the M_1 receptor in activating its

TABLE 1

Parameters of [^{35}S]GTPγS binding to membranes from CHO cells

E_{MAX} is expressed as percentage of increase above basal binding, and concentrations of agonists producing EC_{50} are expressed as negative logarithms of [^{35}S]GTPγS binding to membranes from CHO cells expressing M_1 or M_2 receptors, respectively. Data are means ± S.E.M. from four to six independent experiments performed in quadruplicates.

	M_1		M_2	
	pEC_{50}	E_{MAX}	pEC_{50}	E_{MAX}
		% above basal		*% above basal*
Without preincubation				
Xanomeline	8.04 ± 0.01*	228 ± 9*	N.E.	N.E.
Carbachol	6.08 ± 0.01	179 ± 7	6.12 ± 0.05	187 ± 0.06
+ 3 nM xanomeline	5.97 ± 0.03*	179 ± 8	N.M.	N.M.
+ 10 nM xanomeline	5.79 ± 0.04*	183 ± 7	6.01 ± 0.05	186 ± 0.06
+ 30 nM xanomeline	5.39 ± 0.01*	184 ± 8	5.89 ± 0.03*	185 ± 0.09
+ 100 nM xanomeline	5.02 ± 0.02*	184 ± 9	5.63 ± 0.02*	182 ± 0.08
+ 300 nM xanomeline	N.M.	N.M.	5.16 ± 0.05*	196 ± 0.09
With 60-min preincubation				
Xanomeline	8.15 ± 0.04*	224 ± 6*	7.78 ± 0.03	75 ± 5*
Carbachol	6.08 ± 0.04	189 ± 6	6.12 ± 0.03	188 ± 6
+ 3 nM xanomeline	5.92 ± 0.04*	189 ± 7	N.M.	N.M.
+ 10 nM xanomeline	5.68 ± 0.03*	189 ± 8	6.02 ± 0.01	191 ± 7
+ 30 nM xanomeline	5.32 ± 0.03*	195 ± 9	5.68 ± 0.02*	197 ± 8
+ 100 nM xanomeline	4.91 ± 0.03*	194 ± 11	5.28 ± 0.03*	190 ± 8
+ 300 nM xanomeline	N.M.	N.M.	4.97 ± 0.04*	194 ± 7
With 60-min preincubation followed by washing				
Xanomeline	5.98 ± 0.03	208 ± 6*	5.81 ± 0.03*	62 ± 5*
Carbachol	6.07 ± 0.03	186 ± 6	6.06 ± 0.03	195 ± 6
+ 0.3 μM xanomeline	6.08 ± 0.04	189 ± 7	6.04 ± 0.04	190 ± 8
+ 1 μM xanomeline	6.18 ± 0.01	187 ± 9	6.07 ± 0.05	181 ± 9
+ 3 μM xanomeline	5.84 ± 0.05*	194 ± 8	6.01 ± 0.02	173 ± 7*
+ 10 μM xanomeline	5.42 ± 0.05*	178 ± 9	5.86 ± 0.01*	165 ± 7*

N.E., no effect; N.M., not measured.
* $P < 0.05$, significantly different from carbachol or carbachol alone by t test or ANOVA followed by Dunnett's test.

TABLE 2

Estimates of xanomeline pK_B in antagonizing the responses to carbachol

Negative logarithms of xanomeline pK_B based on its effect on carbachol-stimulated [^{35}S]GTPγS binding. Constants were obtained by nonlinear regression of eq. 5 to EC_{50} of carbachol concentration-response curves from individual experiments. Table shows averages ± S.E.M. (n = 4). Values of pEC_{50} of xanomeline concentration-response curves and pK_I of xanomeline inhibition of [^{3}H]NMS binding are shown for comparison.

	M_1			M_2		
	pK_B	pEC_{50}	pK_I	pK_B	pEC_{50}	pK_I
Without preincubation	8.08 ± 0.04*	8.04 ± 0.01*	7.87 ± 0.04	7.37 ± 0.03	N.E.	7.43 ± 0.04
60-min preincubation	8.15 ± 0.02†	8.15 ± 0.04†	N.M.	7.77 ± 0.04†	7.78 ± 0.03	N.M.
60-min preincubation followed by washing	5.69 ± 0.04*‡	5.98 ± 0.03*	6.53 ± 0.05	4.78 ± 0.03*‡	5.81 ± 0.03*	6.53 ± 0.05

N.E., no effect; N.M., not measured.
* Significantly different from corresponding pK_I by ANOVA followed by Tukey-Kramer post-test.
† Significantly different from without preincubation by ANOVA followed by Tukey-Kramer post-test.
‡ Significantly different from corresponding pEC_{50} by ANOVA followed by Tukey-Kramer post-test.

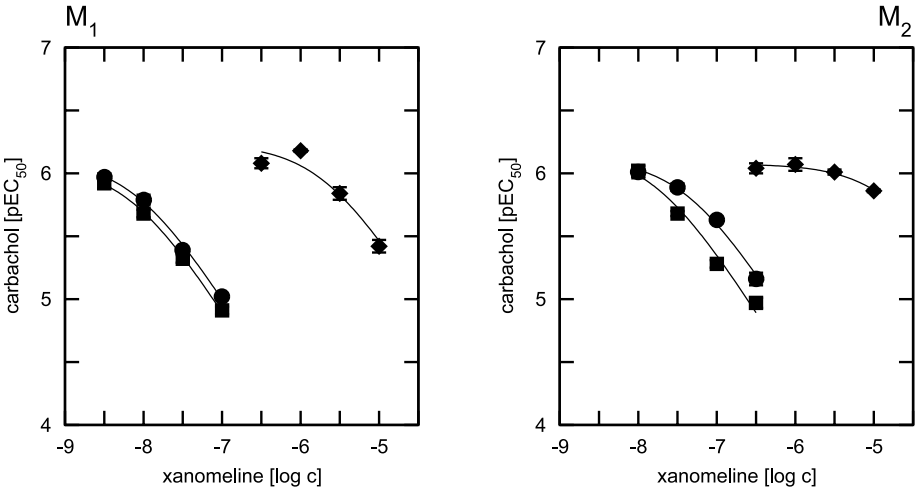

Fig. 6. Clark plots of xanomeline effects on carbachol stimulation of [^{35}S]GTPγS binding to M_1 and M_2 CHO membranes. Negative logarithm of half-maximal concentration (pEC_{50}) of carbachol-stimulated [^{35}S]GTPγS binding (ordinate) to M_1 (left) or M_2 (right) CHO membranes is plotted as a function of xanomeline concentration (abscissa, log M). ●, xanomeline was added simultaneously with [^{35}S]GTPγS. ■, xanomeline was added 60 min ahead of [^{35}S]GTPγS; ◆, membranes were preincubated for 60 min with xanomeline in the concentrations indicated on the x-axis and washed before addition of carbachol and [^{35}S]GTPγS. Curves are fits of eq. 5 to the data. Data are means ± S.E.M of four independent experiments performed in quadruplicate.

principal G_q subtype, it was only 35 times more potent at the M_2 receptor in activating its preferred G protein subtype, G_i (Table 3). Differences in potencies between xanomeline and carbachol at the remaining G protein subtypes were much smaller, being only in the range of 3 to 8 times. These observations provide evidence that xanomeline is better than carbachol in discriminating among G protein subtypes and that this discrimination is more marked at the M_1 than at the M_2 receptor.

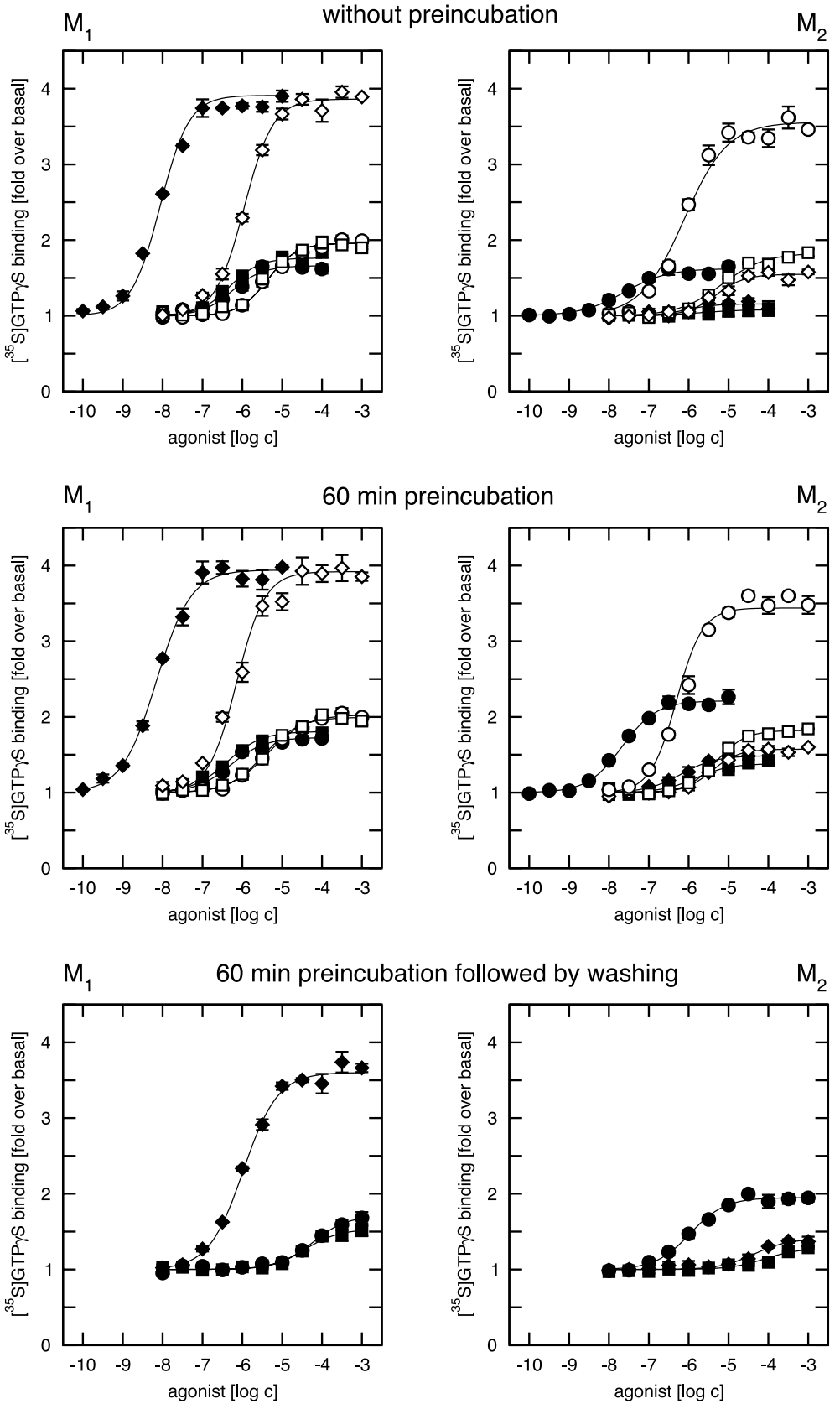

Fig. 7. Stimulation of [^{35}S]GTPγS binding to G_i, G_s, and G_q G proteins by carbachol and xanomeline. [^{35}S]GTPγS binding (ordinate, -fold increase over basal) to G_i (circles), G_s (squares), and G_q (diamonds) G protein α subunits in CHO membranes expressing M_1 (left) or M_2 (right) receptors stimulated by carbachol (open symbols) or xanomeline (closed symbols) was determined using scintillation proximity assay. Top row, agonists were added simultaneously with [^{35}S]GTPγS. Middle row, agonists were added 60 min ahead of [^{35}S]GTPγS. Bottom row, membranes were preincubated with increasing concentrations of xanomeline as indicated on the *x*-axis and washed before incubation with [^{35}S]GTPγS. Data are means ± S.E.M. of three to six independent experiments performed in quadruplicate.

Wash-resistant xanomeline binding at both receptors was accompanied by stimulation of [^{35}S]GTPγS binding to all tested subtypes of G proteins. Potencies of xanomeline in activating all G protein subtypes at both receptors were approximately 100 times lower than the values obtained in the continued presence of xanomeline. Efficacies of xanomeline in stimulating G_q at M_1 receptors and G_i at M_2 receptors after washing were 88 and 79%, respectively, of levels observed after preincubation without washing. Furthermore, washing decreased efficacies of xanomeline for nonprincipal G proteins to 68% (G_i) and 65% (G_s) at M_1 receptors but only to 73% (G_s) and 83% (G_q) at M_2 receptors compared with nonwashing conditions (Table 3). However, effects of washing were statistically significant only in the case of M_1 receptors.

Discussion

Our data demonstrate that xanomeline binds to both the M_1 and M_2 subtypes of muscarinic acetylcholine receptors in a wash-resistant manner, albeit with an apparent lower potency than reversible binding. These observations confirm and complement previous reports demonstrating wash-resistant xanomeline binding at M_1 and M_5 muscarinic receptors, accompanied by persistent receptor activation and antagonism, respectively (Christopoulos et al., 1998; Grant and El-Fakahany, 2005). We have previously provided experimental evidence that persistent attachment of xanomeline develops at receptor domains distinct from the orthosteric agonist binding site (Christopoulos et al., 1998, 1999; Jakubík et al., 2002) and depends on the length of *O*-alkyl side chain of xanomeline and the receptor lipid environment (Jakubík et al., 2004).

In the present work, we detected striking differences in the kinetics of xanomeline wash-resistant binding and receptor activation at the M_1 and M_2 receptors. Development of xanomeline wash-resistant binding to M_2 receptors is markedly slower than to M_1 receptors (Fig. 2). Most obviously, xanomeline does not display at the M_2 receptor the instant wash-resistant binding component it shows at the M_1 receptor. Analysis of the relationship between xanomeline concentration and the rate of appearance of its wash-resistant binding to M_1 and M_2 receptors revealed marked deviation from expected features of a simple bimolecular interaction (Fig. 3). This deviation was more marked in the case of the M_1 receptor.

Given the fast rate of xanomeline wash-resistant binding, one has to assume that quantification of the interaction of free xanomeline interactions with the receptor is less straightforward because of the involvement of both reversible and wash-resistant binding components. Nonetheless, several pieces of evidence indicate that the interaction of free xanomeline during its continuous presence is competitive in nature, at both M_1 and M_2 receptors. First, xanomeline decreases the potency of carbachol in activating the receptors without a change in its efficacy (Fig. 4, top and middle; Table 1). Second, there is close correspondence of the potency of xanomeline when continuously present in inhibiting [^{3}H]NMS binding and in attenuating the carbachol response (Fig. 5; Table 1), in spite of some deviations. Third, there is good agreement in the values of affinity of xanomeline interaction with the receptors as calculated by its ability to activate the receptor, inhibit [^{3}H]NMS binding, and shift the carbachol concentration-response curve (Table 2). The apparent competitive interaction of free xanomeline is in line with previous reports by our group, namely, the demonstration of

TABLE 3

Parameters of induced [^{35}S]GTPγS binding to G_i, G_s, and G_q subtypes of G proteins

Induced binding to individual subtypes of G proteins in membranes from CHO cells expressing M_1 or M_2 receptors, respectively, was detected by scintillation proximity assay. E_{MAX} is expressed as percentage increase above basal binding, and concentrations of agonist producing EC_{50} are expressed as negative logarithms of [^{35}S]GTPγS binding to G_i, G_s, and G_q G proteins, respectively. Data are means ± S.E.M. from three to four independent experiments performed in quadruplicates.

	M_1		M_2	
	pEC_{50}	E_{MAX}	pEC_{50}	E_{MAX}
		% above basal		*% above basal*
Without preincubation				
Carbachol				
G_i	5.33 ± 0.06	96 ± 7	6.11 ± 0.05	248 ± 11
G_s	5.44 ± 0.05	92 ± 8	5.29 ± 0.06	79 ± 6
G_q	5.94 ± 0.06	289 ± 11	5.38 ± 0.05	55 ± 5
Xanomeline				
G_i	6.28 ± 0.06	68 ± 6	7.54 ± 0.05	59 ± 6
G_s	6.37 ± 0.04	77 ± 7	n.e.	n.e.
G_q	8.11 ± 0.04	283 ± 9	n.e.	n.e.
With 60-min preincubation				
Carbachol				
G_i	5.37 ± 0.05	103 ± 8	6.13 ± 0.05	255 ± 12
G_s	5.46 ± 0.04	100 ± 7	5.31 ± 0.06	82 ± 6
G_q	6.01 ± 0.04	309 ± 12	5.42 ± 0.04	57 ± 6
Xanomeline				
G_i	6.30 ± 0.05	72 ± 7	7.67 ± 0.06*	121 ± 7*
G_s	6.39 ± 0.05	81 ± 7	5.82 ± 0.04	38 ± 4
G_q	8.16 ± 0.03	294 ± 10	6.19 ± 0.04	49 ± 4
With 60-min preincubation followed by washing				
Xanomeline				
G_i	4.23 ± 0.05†	49 ± 4†	5.96 ± 0.06†	95 ± 6†
G_s	4.43 ± 0.05†	53 ± 4†	4.09 ± 0.05†	28 ± 5
G_q	5.98 ± 0.04†	260 ± 10	4.36 ± 0.05†	41 ± 5

n.e., no effect.

* Significantly different from no preincubation ($P < 0.05$; ANOVA followed by Tukey-Kramer post-test).

† Significantly different from 60-min preincubation ($P < 0.05$; ANOVA followed by Tukey-Kramer post-test).

only its competitive binding to purified M_1 receptors (Jakubík et al., 2004).

The instantaneous formation of xanomeline wash-resistant inhibition of [^{3}H]NMS binding at the M_1 receptor was not accompanied by receptor activation. This difference cannot be explained by the possible presence of receptor reserve in the [^{35}S]GTPγS signal. If anything, receptor reserve would be expected to result in reaching a maximal response at a rate faster than that of receptor occupation, resulting in *overestimation* of the rate of receptor activation. Moreover, coaddition of xanomeline and [^{35}S]GTPγS in case of the M_2 receptor (no preincubation conditions; Fig. 6, top right) results in shifting the carbachol concentration-response curve to the right in the absence of receptor activation by xanomeline. These differential temporal effects of xanomeline probably represent time-dependent transitions of the conformation of its complex with the receptor or time-dependent binding to different domains on the receptor.

Another difference in the interaction of wash-resistant xanomeline with the M_1 and the M_2 receptors in the presence of free ligand occurs in its differential efficacy in activating [^{35}S]GTPγS binding. Whereas xanomeline, after 60-min preincubation, acts as a potent partial agonist at M_2 receptors, it behaves like a potent full agonist at the M_1 receptor (Fig. 5; Table 1). Indeed, xanomeline produces a higher maximal response at the M_1 receptor than the conventional full agonist carbachol. Thus, xanomeline might be considered as a super agonist at M_1 receptors. In agreement with the notion of partial agonistic activity of xanomeline at M_2 receptors, its efficacy in enhancing [^{35}S]GTPγS binding can be augmented by increasing the receptor expression level (data not shown).

In contrast, interaction between wash-resistant xanomeline binding in the absence of free ligand and the M_1 or the M_2 receptors seems more complex. Pretreatment with xanomeline at concentrations up to 1 μM at M_1 receptors or up to 3 μM at M_2 receptors followed by washing does not shift carbachol concentration-response curves of [^{35}S]GTPγS binding (Fig. 5, bottom), despite its binding to the receptors as evidenced by attenuation of [^{3}H]NMS binding and activation of the receptors. This discrepancy suggests that lower concentrations of xanomeline bind to the receptor in a wash-resistant manner without interfering with the ability of carbachol to interact with the receptor and activate it. This interpretation is in concert with our previously suggested models of interaction of xanomeline with muscarinic receptors (Jakubík et al., 2002) and activation of muscarinic receptors by allosteric modulators (Jakubík et al., 1996) or small ectopic ligands (Spalding et al., 2002). A common feature of the proposed models is that these compounds interact with receptor domains different from the orthosteric binding site. Pretreatment with higher concentrations of xanomeline followed by washing decreases the potency of carbachol in activating M_1 and M_2 receptors, indicating attenuation of the affinity of carbachol binding to the receptor. However, wash-resistant bound xanomeline is clearly more potent in displacing [^{3}H]NMS binding than at shifting the carbachol concentration-response curves at both receptors (Fig. 5; Table 2). This suggests that xanomeline binds avidly to the receptor in a manner that differentially influences agonist and antagonist binding. The observed more marked discrepancy in the effects of xanomeline on antagonist binding and agonist response at the M_2 receptor subtype suggests differences in the conformation of the two receptors when persistently occupied by xanomeline. Alternatively, wash-resistant xanomeline binding takes place at different domains on the two receptors, with varying distances from the agonist orthosteric binding site on the receptor. The observed marked differences in the kinetics of wash-resistant xanomeline binding at the two receptors and the observed reduction of the maximal response to carbachol only at the M_2 receptor (Table 1) support either conclusion.

Furthermore, wash-resistant xanomeline binding is more potent in activating M_1 or M_2 receptors than in influencing the response to carbachol. Thus, formation of xanomeline wash-resistant binding might transit through several steps. This transition ends with at least two interchangeable functionally active states; one that does not affect carbachol action and another that attenuates receptor stimulation by carbachol. Different ratios of xanomeline pEC_{50} to pK_B at M_1 and M_2 receptors (Table 2) indicate preferential predominance of these two putative binding states at the two subtypes of muscarinic receptors. Together, this interpretation is in line with our proposal of the existence of multiple interchangeable binding modes of xanomeline binding with muscarinic receptors (Jakubík et al., 2002).

We have also shown other important differences in the receptor agonistic effects of xanomeline and the conventional full agonist carbachol regarding effecting coupling of M_1 and M_2 receptors to various subtypes of G proteins. Namely, xanomeline is more selective in its efficacy than carbachol in favoring coupling of each receptor subtype to its preferred G protein (G_q in M_1 and G_i in M_2) in comparison with other G proteins (Table 3). Xanomeline also exhibits a higher potency ratio than carbachol in activating preferred versus nonpreferred G proteins. This indicator of xanomeline selectivity is more pronounced at M_1 than at M_2 receptors. Thus, xanomeline distinguishes between G protein subtypes better than carbachol, with more discrimination at M_1 than at M_2 receptors. Distinction by xanomeline among different G proteins is maintained after washing of the free drug. Our observations support the existence of multiple active receptor conformations that differ in affinities for individual G protein subtypes. Different agonists favor certain active receptor conformations over others. This is in agreement with the concept of agonist trafficking (Kenakin, 1995).

In summary, xanomeline demonstrates marked selectivity in its binding kinetics and agonistic activity at M_1 and M_2 muscarinic receptors. The latter is evident in better ability of xanomeline than carbachol to differentiate between coupling of the receptor to various G proteins, in terms of both efficacy and potency. Such differences may work in concert to contribute to known functional selectivity of xanomeline toward M_1 over M_2 receptors (Wood et al., 1999). Our data also add further support to the notion that xanomeline is capable of interacting with multiple sites on the muscarinic receptor. This results in divergent conformations of the receptor that vary in their state of activation. These receptor states also induce differential effects on ligand binding to and activation of the receptor orthosteric site. Distribution of such receptor states is both time- and concentration-dependent and varies between the M_1 and the M_2 subtypes of muscarinic receptors. Our observations provide additional support to the hypothesis of agonist trafficking, where different agonists favor cer-

tain active receptor conformations over others (Kenakin, 1995).

References

Bartus RT, Dean RL, and Beer B (1982) Neuropeptide effects on memory in aged monkeys. *Neurobiol Aging* **3:**61–68.

Bonner Ti, Buckley NJ, Young AC, and Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. *Science (Wash DC)* **237:**527–532.

Bymaster FP, Carter PA, Peters SC, Zhang W, Ward JS, Mitch CH, Calligaro DO, Whitesitt CA, DeLapp N, Shannon HE, et al. (1998) Xanomeline compared to other muscarinic agents on stimulation of phosphoinositide hydrolysis in vivo and other cholinomimetic effects. *Brain Res* **795:**179–190.

Bymaster FP, Whitesitt CA, Shannon HE, DeLapp N, Ward JS, Calligaro DO, Shipley LA, Buelke-Sam JL, Bodick NC, Farde L, et al. (1997) Xanomeline: a selective muscarinic agonist for the treatment of Alzheimer's disease. *Drug Dev Res* **40:**158–170.

Caulfield MP (1993) Muscarinic receptors – characterization, coupling and function. *Pharmacol Ther* **58:**319–379.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which cause 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Christopoulos A, Parsons AM, and El-Fakahany EE (1999) Pharmacological analysis of the novel mode of interaction between xanomeline and the M_1 muscarinic acetylcholine receptor. *J Pharmacol Exp Ther* **289:**1220–1228.

Christopoulos A, Pierce TL, Sorman JL, and El-Fakahany EE (1998) On the unique binding and activating properties of xanomeline at the M_1 muscarinic acetylcholine receptor. *Mol Pharmacol* **53:**1120–1130.

DeLapp NW, McKinzie JH, Sawyer BD, Vandergriff A, Falcone J, McClure D, and Felder CC (1999) Determination of [^{35}S]guanosine-5γ-*O*-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. *J Pharmacol Exp Ther* **289:**946–955.

Dolezal V and Kasparova J (2003) β-Amyloid and cholinergic neurons. *Neurochem Res* **28:**499–506.

Francis PT, Palmer AM, Snape M, and Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatr* **66:**137–147.

Grant MKO and El-Fakahany EE (2005) Persistent binding and functional antagonism by xanomeline at the muscarinic M_5 receptor. *J Pharmacol Exp Ther* **315:**313–319.

Jakubík J, Bacakova L, Lisa V, El-Fakahany EE, and Tucek S (1996) Activation of muscarinic acetylcholine receptors via their allosteric binding sites. *Proc Natl Acad Sci USA* **93:**8705–8709.

Jakubík J, Tucek S, and El-Fakahany EE (2002) Allosteric modulation by persistent binding of xanomeline of the interaction of competitive ligands with the M_1 muscarinic acetylcholine receptor. *J Pharmacol Exp Ther* **301:**1033–1041.

Jakubík J, Tucek S, and El-Fakahany EE (2004) Role of receptor protein and membrane lipids in xanomeline wash-resistant binding to muscarinic M_1 receptors. *J Pharmacol Exp Ther* **308:**105–110.

Kaumann AJ and Marano M (1982) On equilibrium dissociation constants for complexes of drug receptor subtypes: selective and nonselective interactions of partial agonists with two β-adrenoreceptor subtypes mediating positive chronotropic effects of (−)isoprenaline in kitten atria. *Naunyn-Schmiedeberg's Arch Pharmacol* **219:**216–221.

Kenakin T (1995) Agonist-receptor efficacy II: agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232–238.

Ladner CJ and Lee JM (1998) Pharmacological drug treatment of Alzheimer disease: the cholinergic hypothesis revisited. *J Neuropathol Exp Neurol* **57:**719–731.

Lew MJ and Angus JA (1996) Analysis of competitive agonist-antagonist interactions by nonlinear regression. *Trends Pharmacol Sci* **16:**328–337.

Michal P, Lysikova M, and Tucek S (2001) Dual effects of muscarinic M_2 acetylcholine receptors on the synthesis of cyclic AMP in CHO cells: dependence on time, receptor density and receptor agonists. *Br J Pharmacol* **132:**1217–1222.

Perry EK, Gibson PH, Blessed G, Perry RH, and Tomlinson BE (1977a) Neurotransmitter enzyme abnormalities in senile dementia: choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. *J Neurol Sci* **34:**247–265.

Perry EK, Perry RH, Blessed G, and Tomlinson BE (1977b) Necropsy evidence of central cholinergic deficits in senile dementia. *Lancet* **1:**18919.

Shannon HE, Bymaster FP, Calligaro DO, Greenwood B, Mitch CH, Sawyer BD, Ward JS, Wong DT, Olesen PH, Sheardown MJ, et al. (1994) Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M_1 receptors. *J Pharmacol Exp Ther* **269:**271–281.

Spalding TA, Trotter C, Skjaerbaerk N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U, and Brann MR (2002) Discovery of an ectopic activation site on the M_1 muscarinic receptor. *Mol Pharmacol* **61:**1297–1302.

Tucek S, Michal P, and Vlachova V (2002) Modelling the consequences of receptor-G-protein promiscuity. *Trends Pharmacol Sci* **23:**171–176.

Ward JS, Merritt L, Calligaro DO, Bymaster FP, Shannon HE, Sawyer BD, Mitch CH, Deeter JB, Peters SC, Sheardown MJ, et al. (1995) Functionally selective M1. muscarinic agonists. 3. Side chains and azacycles contributing to functional muscarinic selectivity among pyrazinylazacycles. *J Med Chem* **38:**3469–3481.

Watson J, Brough S, Coldwell M, Gager T, Ho M, Hunter A, Jerman J, Middlemiss D, Riley G, and Brown A (1998) Functional effects of the muscarinic receptor agonist, xanomeline, at 5-HT_1 and 5-HT_2 receptors. *Br J Pharmacol* **125:**1413–1420.

Wood MD, Murkitt KL, Ho M, Watson JM, Brown F, Hunter AJ, and Middlemiss DN (1999) Functional comparison of muscarinic partial agonists at muscarinic receptor subtypes hM_1, hM_2, hM_3, hM_4 and hM_5 using microphysiometry. *Br J Pharmacol* **126:**1620–1624.

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