Differences in kinetics of xanomeline binding and selectivity of activation of G proteins at M₁ and M₂ muscarinic acetylcholine receptors

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Running title
Xanomeline action at M₁ and M₂ muscarinic receptors

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Abbreviations
CHO, Chinese hamster ovary; GTPγS, guanosine-5′-γ-S-thiotrisphosphate;
Abstract

Xanomeline is a functionally selective M1/M4 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 M1 receptors characterized by persistent binding and receptor activation after extensive washout has been previously shown. In the present study, using human M1 and M2 receptors expressed in Chinese hamster ovary cells and 3H-N-methylscopolamine as a tracer, we show that persistent binding of xanomeline also occurs at the M2 receptor with similar affinity as at the M1 receptor (K_i = 294 and 296 nM, respectively). However, kinetics of formation of xanomeline wash-resistant binding to M2 receptors were markedly slower than to M1 receptors. Xanomeline was a potent fast-acting full agonist in stimulating guanosine-5'-γ[35S]thiotrisphosphate binding at M1 receptors while at M2 receptors it behaved as a potent partial agonist (40% of carbachol maximal response) only upon preincubation for one hour. Development of xanomeline agonistic effects at the M2 receptor was slower than its ability to attenuate carbachol responses. We also demonstrate that xanomeline discriminates better between G-protein subtypes at M1 than at M2 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 M1 and the M2 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); 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; 1977b; 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 Alzheimer’s patients 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-thiadiao-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine) that 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 known.

A remarkable feature of xanomeline action is its ability to stimulate M₁ muscarinic receptors even after intensive washing to remove the free ligand (Christopoulos et al., 1998, 1999). It has been demonstrated that xanomeline binds to M₁ 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 receptor’s orthosteric site in a complex manner (Jakubik et al., 2002). However, wash resistant binding of xanomeline as such cannot explain its functional selectivity because it is not confined to the M₁ receptor subtype. A similar mode of xanomeline binding to the M₅ 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 M₁ receptor where xanomeline behaves as a potent and efficacious agonist, and the M₂ receptor where xanomeline binds equally well but does not result in receptor activation. M₁ 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 (Jakubik 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'-\(\gamma^{[35S]}\)thiotrisphosphate (\([^{35S}]\)GTP\(\gamma\)S) and anti-rabbit IgG coated scintillation proximity beads were from Amersham (UK). Carbachol, dithiotreitol, guanosine-5'-bis-phosphate (GDP), guanosine-5'-\(\gamma\)S-thiotrisphosphate (GTP\(\gamma\)S) and N-methylscopolamine chloride (NMS) were from Sigma (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 M1 or M2 muscarinic receptor genes were grown to confluence in 50 cm\(^2\) flasks in Dulbecco Eagle’s modified medium supplemented with 10% fetal bovine serum and were subcultured to sixteen 100 mm Petri dish (approx. 2 million cells per 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 x g. Washed cells were diluted in ice cold homogenization medium (100 mM NaCl, 20 mM Na-HEPES, 10 mM EDTA; pH = 7.4) and homogenized on ice by two 30 sec strokes using a Polytron homogenizer (Ultra-Turrax; Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen, Germany) with a 30-sec pause between strokes. Cell homogenate was centrifuged for 30 min at 30,000 x g. The supernatant was discarded and pellets resuspended in incubation medium (100 mM NaCl, 10 mM MgCl\(_2\), 20 mM Na-HEPES pH = 7.4) and centrifuged for 30 min at 30,000 x 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. On the other hand, in experiments referred to as "prelabelling / 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 x g at 4°C, and resuspended in incubation medium. Centrifugation and resuspension was 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 $[^{35}\text{S}]{\text{GTP}}\gamma{S}$ (Fig. 2 and 4) to expedite first steps of washing. Cells were treated with xanomeline at 30 °C for the indicated times then centrifuged for 1 min at 300 x 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 addition of EDTA (10 mM) 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 dithiotreitol 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 $[^{3}\text{H}]{\text{NMS}}$. 

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Nonspecific binding was determined in the presence of 10 µM NMS. Incubation with \[^3\text{H}]\text{NMS}\) lasted for 60 min and was terminated by filtration on glass fiber filters. For determination of \[^{35}\text{S}\]\text{GTP}\gamma\text{S}\ binding to G-proteins in membranes a final concentration of 200 pM (M\(_1\) receptors) or 500 pM (M\(_2\) receptors) of \[^{35}\text{S}\]\text{GTP}\gamma\text{S}\ was used, supplemented by 5 µM (M\(_1\) receptors) or 50 µM (M\(_2\) receptors) GDP. \[^{35}\text{S}\]\text{GTP}\gamma\text{S}\ nonspecific binding was determined in the presence of 1 µM \text{GTP}\gamma\text{S}. Incubation with \[^{35}\text{S}\]\text{GTP}\gamma\text{S}\ was carried for 20 min and free ligand was removed by filtration through Whatman GF/F glass fiber filters using a Tomtech Mach III cell harvester. Filters were dried in vacuum for 1 h while heated at 80 °C, then solid scintillator Meltilex A was melted on filters (105 °C, 90 sec) using a hot plate. After cooling the filters were counted using a Wallac Microbeta scintillation counter.

**Scintillation proximity assay**

In case of scintillation proximity assay incubation with \[^{35}\text{S}\]\text{GTP}\gamma\text{S}\ 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 alpha subunits were added and incubation was continued for 1 h. The final dilution was 1:1000 in case of anti-G\(_i\)-alpha and anti-G\(_s\)-alpha antibodies and 1:2000 in case of the anti-G\(_q\)-alpha 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 1,000 x g and counted using the scintillation proximity assay protocol in a Wallac Microbeta scintillation counter.
Data analysis

Data were preprocessed by Open Office 1.1.5 (www.openoffice.org) and subsequently analyzed by Grace 5.1.18 (plazma-gate.wezman.ac.il) and statistic package R (www.r-project.org) on Mandriva distribution of Linux.

The following equations were fitted to data:

Interference with [³H]NMS binding

\[ y = 100 \times \left(1 - \frac{x}{IC_{50}^{nH} + x}\right) \]  \hspace{1cm} (Eq. 1)

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

Time-dependent decrease in [³H]NMS binding by xanomeline

\[ y = (100 - \text{Plateau})e^{-K_{obs}x} + \text{Plateau} \]  \hspace{1cm} (Eq. 2)

\( y \), binding of [³H]NMS at time \( x \) normalized to its binding at time 0; plateau, normalized [³H]NMS binding at steady state; \( K_{obs} \), observed rate of xanomeline binding.
Time-dependent increase in $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding

$$y = Y_0 + Y_{\text{MAX}}^* (1 - e^{-Kx}) \quad (\text{Eq. 3})$$

$y$, binding of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ at time $x$; $Y_0$, binding at time 0; $Y_{\text{MAX}}$, maximum binding; $K$, rate constant.

Concentration-response enhancement of $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding by agonists

$$y = 100 + E_{\text{MAX}} / (1 + (EC_{50} - x)^{1/nH}) \quad (\text{Eq. 4})$$

$y$, $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding in the presence of agonist at concentration $x$ normalized to binding in the absence of agonist; $E_{\text{MAX}}$, maximal per cent increase by agonist; $EC_{50}$, concentration of agonist producing 50% of maximal effect; $nH$, Hill coefficient.

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

$$pEC_{50} = -\log([x] + 10^{-pKB}) - \log c \quad (\text{Eq. 5})$$
pEC$_{50}$, negative logarithm of agonist EC$_{50}$ in the presence of competitor at concentration x; pK$_{B}$, negative logarithm of equilibrium dissociation constant of competitor; c, fitting constant.
Results

Xanomeline reversible and wash-resistant binding to M₁ and M₂ muscarinic receptors

Experiments were performed on membranes from CHO cells stably expressing M₁ and M₂ receptors (1.8 ± 0.1 and 1.2 ± 0.1 pmol of [³H]NMS binding sites per mg of protein in membranes, respectively). The equilibrium dissociation constant (Kd) of [³H]NMS to CHO-M₁ and CHO-M₂ membranes was 418 ± 20 and 524 ± 25 pM, respectively, and was the same under all experimental setups. Affinity of interaction of xanomeline with M₁ and M₂ muscarinic receptors was determined by its ability to displace binding of 1 nM [³H]NMS to membranes of CHO cells that stably express each receptor subtype (Fig. 1). The equilibrium dissociation constant (Kᵢ) of xanomeline at the M₁ and M₂ muscarinic receptors was 13.5 ± 1.5 and 37.2 ± 4.1 nM, respectively. Membranes pretreated with xanomeline for 60 minutes followed by extensive washing exhibited concentration-dependent reduction in subsequent binding of [³H]NMS in the absence of free xanomeline, albeit with lower potency of xanomeline in comparison to that obtained in its continuous presence in the binding assay medium (Fig. 1). The Kᵢ of xanomeline wash-resistant binding to M₁ and M₂ muscarinic receptors was 296 ± 31 and 294 ± 34 nM, respectively. These results thus indicate slightly higher affinity of reversible xanomeline binding to M₁ than M₂ receptors while affinity of its wash-resistant binding is the same for both subtypes.

Kinetics of xanomeline wash-resistant binding to M₁ and M₂ muscarinic receptors

To detect possible differences between M₁ and M₂ receptors in the rate of formation of xanomeline wash-resistant binding cells were exposed to xanomeline for various periods
of time then washed and incubated with [³H]NMS. Decrease in [³H]NMS binding was taken as measure of xanomeline wash-resistant binding. Formation of xanomeline wash-resistant binding at M₁ receptors was extremely fast and already appeared upon washing cells immediately following addition of xanomeline (Fig. 2 left; time 0). Observed association constants (K_{obs}) of this interaction did not follow the concentration dependency 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 previously reported (Jakubik et al., 2002). In contrast, formation of xanomeline wash-resistant binding at M₂ receptors was markedly slower than at M₁ receptors (Fig. 2 right). Most notably, there was no evidence of the instantaneous phase of xanomeline wash-resistant binding shown with M₁ 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 M₂ receptor. For example, increasing xanomeline concentration from 0.3 to 10 µM changed K_{obs} almost by 10 fold. However, the relationship between xanomeline concentration and K_{obs} at the M₂ receptor still deviated from a simple bimolecular scheme of interaction (Fig. 3), even though the deviation was less marked than in the case of the M₁ receptor.

Kinetics of xanomeline wash-resistant activation of M₁ and M₂ receptors

In related experiments we explored possible differences in the rate of xanomeline wash-resistant activation of M₁ and M₂ receptors. Membranes were exposed to xanomeline for
various periods of time then washed and incubated with \([^{35}\text{S}]\text{GTP}\gamma\text{S}\). Increase in \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding was taken as a measure of receptor activation by persistently-bound xanomeline. Preincubation with 10 \(\mu\text{M}\) xanomeline followed by washing resulted in a time-dependent activation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding at M1 and M2 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 M1 and M2 receptors, xanomeline exhibited slower wash-resistant activation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding at the M2 receptor. Appearance of xanomeline wash-resistant activation of muscarinic receptors was significantly slower than the formation of xanomeline wash-resistant inhibition of \([^{3}\text{H}]\text{NMS}\) binding (\(P<0.05\), unpaired t-test). For example, while preincubation with 10 \(\mu\text{M}\) xanomeline resulted in an instantaneous 35\% wash-resistant decrease in \([^{3}\text{H}]\text{NMS}\) binding at M1 receptors (Fig. 2), there was no corresponding enhancement of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding (Fig. 4). Furthermore, while wash-resistant binding of 10 \(\mu\text{M}\) xanomeline to the M1 receptor reached equilibrium at 10 min, \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) 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 M2 receptor and persistent activation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding were 0.318 ± 0.021 min\(^{-1}\) 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 [35S]GTPγS binding to membranes. Due to the complex nature of xanomeline binding it was necessary to employ different experimental setups: 1/ Simultaneous addition of xanomeline or carbachol or their combination and [35S]GTPγS to measure immediate effects of xanomeline. 2/ Preincubation of membranes with xanomeline for 60 min preceding incubation with [35S]GTPγS, with or without carbachol. In this setup both immediate as well as delayed effects of xanomeline are measured. 3/ Preincubation with xanomeline for 60 min followed by washing and incubation with [35S]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 [35S]GTPγS binding are shown in Fig. 5. Curve parameters obtained by fitting Eq. 4 to data from individual experiments are summarized in Table1. In concert with reported functional selectivity of xanomeline, simultaneous addition of xanomeline and [35S]GTPγS stimulated [35S]GTPγS binding at M1 but not at M2 receptors. In contrast, carbachol stimulated [35S]GTPγS binding at both subtypes with the same potency and efficacy (Fig. 5, top and Table 1). At M1 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 [35S]GTPγS binding gradually decreased in the presence of increasing concentrations of xanomeline without a change in carbachol efficacy.
At the M₁ receptor, preincubation in the presence of agonists for 60 min before the addition of [³⁵S]GTPγS resulted in similar effects of xanomeline, carbachol, and their combination on [³⁵S]GTPγS binding as compared to simultaneous addition of the agonists with [³⁵S]GTPγS (Fig. 5 middle). At the M₂ receptor, however, a small but significant stimulatory effect of xanomeline was observed (pEC₅₀ 7.78 ± 0.03, EMAX 1.75 ± 0.05 fold over basal), in contrast to the lack of agonistic activity upon simultaneous addition of xanomeline and [³⁵S]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 minutes followed by extensive washing resulted in concentration-dependent enhancement of [³⁵S]GTPγS binding at both M₁ or M₂ receptors (Fig. 5). Xanomeline wash-resistant receptor activation exhibited the same efficacy at both receptors as in preincubation with xanomeline before addition of [³⁵S]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 M₁ and the M₂ 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 M₁ or M₂ receptors in spite of causing wash-resistant receptor activation (Fig. 5 bottom and Table 1). Thus, the potency of persistently bound xanomeline is higher at causing receptor activation and at decreasing [³⁵H]NMS binding to the receptor than at antagonism of receptor activation by carbachol. In addition, in the
preincubation/washing procedure xanomeline (3 µM and 10 µM) decreased the maximal response to carbachol at M₂ but not at M₁ 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.

Effects of xanomeline on carbachol concentration-response curves were therefore analyzed by Clark’s non-linear regression as described by Lew and Angus (1996). In this analysis pEC₅₀ values obtained by fitting Eq. 4 to carbachol concentration-response curves in the presence of xanomeline (Fig. 5, closed symbols and 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\textsubscript{50}). However, for the preincubation/washing procedure the estimated xanomeline K\textsubscript{B} as an antagonist is lower than its EC\textsubscript{50} values as an agonist at both M\textsubscript{1} and M\textsubscript{2} receptors. However, while at M\textsubscript{1} receptors K\textsubscript{B} of xanomeline is only two times higher than its EC\textsubscript{50}, the corresponding ratio is more than 10 fold at the M\textsubscript{2} receptor. Preincubation with xanomeline caused lowering of its K\textsubscript{B} at the M\textsubscript{2} receptor by 2.5 fold (enhanced affinity), but did not alter the corresponding value at the M\textsubscript{1} receptor. Washing xanomeline out following preincubation resulted in marked increases in K\textsubscript{B} values. Thus, washing reduced the potency of xanomeline in antagonizing the effects of carbachol by 300 and 1000 fold at M\textsubscript{1} and M\textsubscript{2} receptors, respectively.

 Activation of various G proteins by xanomeline and carbachol at M\textsubscript{1} and M\textsubscript{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 $[^{35}\text{S}]$GTP\textsubscript{γS} binding by carbachol or xanomeline to the G\textsubscript{i}, G\textsubscript{s} and G\textsubscript{q} subtypes of G-proteins at M\textsubscript{1} or M\textsubscript{2} receptors. At M\textsubscript{1} receptors, simultaneous addition of either carbachol or xanomeline with $[^{35}\text{S}]$GTP\textsubscript{γS} preferentially activated G\textsubscript{q} with equal high efficacy. Both agonists also activated G\textsubscript{i} and G\textsubscript{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\textsubscript{q} on the one hand and G\textsubscript{s} and G\textsubscript{i} on the other hand. A similar pattern was observed after 60 min preincubation with agonists prior to the addition of $[^{35}\text{S}]$GTP\textsubscript{γS}. 
Efficacy of xanomeline in stimulating Gi \[^{35}\text{S}]\text{GTP}\gamma\text{S}\] binding at M\(_2\) receptors was only 23\% that of carbachol when added simultaneously with \[^{35}\text{S}]\text{GTP}\gamma\text{S}\] with very small or no stimulation of G\(_s\) or G\(_q\) (Fig. 7, upper right graph and Table 3). Effects of carbachol at all three subtypes of G proteins did not change when it was added 60 min ahead of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\]. However, similar preincubation with xanomeline potentiated its ability to stimulate \[^{35}\text{S}]\text{GTP}\gamma\text{S}\] binding at Gi and also uncovered activation of G\(_s\) and G\(_q\) that was absent when xanomeline 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. While xanomeline present during 60 min preincubation was more than 140-times more potent than carbachol at the M\(_1\) receptor in activating its 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.

Wash resistant xanomeline binding at both receptors was accompanied by stimulation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\] binding to all tested subtypes of G-proteins. Potencies of xanomeline in activating all G-protein subtypes at both receptors were about 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 non-principal 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 to non-washing 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 M₁ and M₂ 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 M₁ and M₅ 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 classical orthosteric agonist binding site (Christopoulos et al., 1998, 1999; Jakubik et al., 2002), and depends on the length of O-alkyl side chain of xanomeline and the receptor lipid environment (Jakubik et al. 2004).

In the present work we detected striking differences in the kinetics of xanomeline wash-resistant binding and receptor activation at the M₁ and M₂ receptors. Development of xanomeline wash-resistant binding to M₂ receptors is markedly slower than to M₁ receptors (Fig. 2). Most obviously, xanomeline does not display at the M₂ receptor the instant wash-resistant binding component it shows at the M₁ receptor. Analysis of the relationship between xanomeline concentration and the rate of appearance of its wash-resistant binding to M₁ and M₂ receptors revealed marked deviation from expected features of a simple bimolecular interaction (Fig. 3). This deviation was more marked in the case of the M₁ 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 due to 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, both at M₁ and M₂ receptors. First, xanomeline decreases the potency of carbachol in activating the receptors without a change in its efficacy (Fig. 4, upper and middle graphs; Tab. 1). Second, there is close correspondence of the potency of xanomeline when continuously present in inhibiting [³H]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 [³H]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 only its competitive binding to purified M₁ receptors (Jakubik et al., 2004).

The instantaneous formation of xanomeline wash-resistant inhibition of [³H]NMS binding at the M₁ receptor was not accompanied by receptor activation. This difference cannot be explained by the possible presence of receptor reserve in the [³⁵S]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, co-addition of xanomeline and [³⁵S]GTPγS in case of the M₂ receptor (no preincubation conditions; Fig 6, upper 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 likely 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 M1 and the M2 receptors in the presence of free ligand appears in its differential efficacy in activating \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding. While xanomeline after 60 min preincubation acts as a potent partial agonist at M2 receptors it behaves like a potent full agonist at the M1 receptor (Fig. 5; Table 1). In fact, xanomeline produces a higher maximal response at the M1 receptor than the conventional full agonist carbachol. Thus, xanomeline might be considered as a super agonist at M1 receptors. In agreement with the notion of partial agonistic activity of xanomeline at M2 receptors, its efficacy in enhancing \[^{35}\text{S}]\text{GTP}\gamma\text{S} 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 M1 or the M2 receptors appears more complex. Pretreatment with xanomeline at concentrations up to 1 \(\mu\text{M}\) at M1 receptors or up to 3 \(\mu\text{M}\) at M2 receptors followed by washing does not shift carbachol concentration-response curves of \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding (Fig. 5 lower graphs) despite its binding to the receptors as evidenced by attenuation of \[^{3}\text{H}]\text{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 (Jakubik et al., 2002) and activation of muscarinic receptors by allosteric modulators (Jakubik 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 classical 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 \( [^3H] \)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 pKB 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. Taken together, this interpretation is in line with our proposal of the existence of multiple interchangeable binding modes of xanomeline binding with muscarinic receptors (Jakubik 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 case of M$_1$ and G$_i$ in case of M$_2$) in comparison to 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. In other words, 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 following 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 M₁ and M₂ muscarinic receptors. The latter is evident in better ability of xanomeline than carbachol to differentiate between coupling of the receptor to various G proteins, both in terms of efficacy and potency. Such differences may work in concert to contribute to xanomeline’s known functional selectivity towards M₁ over M₂ 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 M₁ and the M₂ subtypes of muscarinic receptors. Our observations provide additional support to the hypothesis of agonist trafficking, where different agonists favor certain active receptor conformations over others (Kenakin, 1995).
References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which cause 50 per cent inhibition (IC50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.
MOL #23762


MOL #23762


Footnotes

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Praha, Czech Republic, E-mail: jakubik@biomed.cas.cz
Legends to figures

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 then incubated with $[^{3}H]NMS$ (open symbols). Data are means ± S.E.M. of 3 to 4 independent experiments performed in triplicates. Hill slopes are not significantly different from unity (P<0.05, Wilcoxon test).

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. Circles, 0.3 µM; squares, 1 µM; diamonds 3 µM; triangles 10 µM xanomeline. Data are means ± S.E.M. of 3 independent experiments performed in quadruplicates.

Fig. 3
Comparison of observed rate of loss of $[^3H]NMS$ binding at $M_1$ and $M_2$ muscarinic receptors by xanomeline preincubation.

Observed rate constants $K_{\text{obs}}$ of loss of $[^3H]NMS$ to $M_1$ (circles) or $M_2$ (squares) 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 $K_{\text{obs}}$ based on extrapolation of $K_{\text{obs}}$ measured at 300 nM xanomeline and assuming a simple bimolecular reaction of xanomeline with the receptor.

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 $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding. Squares, 10 µM xanomeline alone; circles, 10 µM xanomeline in the presence of 10 µM NMS. Data are means ± S.E.M. of 3 independent experiments performed in quadruplicates. Observed rate constants are $0.202 \pm 0.013$ and $0.0795 \pm 0.0032 \text{ min}^{-1}$ at $M_1$ and $M_2$ receptors, respectively.

Fig. 5

Stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding by carbachol and xanomeline.

Concentration-response of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding to CHO membranes expressing $M_1$ (Left) or $M_2$ (Right) receptors induced by xanomeline alone (open circles), carbachol alone
(open squares) or carbachol in the continuous presence of increasing concentrations of xanomeline (closed symbols; circles, 3 nM; squares, 10 nM; diamonds, 30 nM; up-triangles, 100 nM; down-triangles 300 nM) or in the preincubation/washing protocol (down-triangles, 300 nM; right-triangles, 1 µM; left-triangles, 3 µM; cross-hair, 10 µM). Upper row, agonists were added simultaneously with $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Middle row, agonists were added 60 min ahead of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Lower row, membranes were preincubated for 60 min with increasing concentrations of xanomeline followed by washing and co-addition of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ with buffer or increasing concentrations of carbachol. Data are means ± S.E.M of 4 to 6 independent experiments performed in quadruplicates.

Fig. 6
Clark plots of xanomeline effects on carbachol stimulation of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding to $M_1$ and $M_2$ CHO membranes.

Negative logarithm of half maximal concentration (pEC$_{50}$) of carbachol-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding (ordinate,) to $M_1$ (left) or $M_2$ (right) CHO membranes is plotted as a function of xanomeline concentration (abscissa, log M). Circles, xanomeline was added simultaneously with $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Squares, xanomeline was added 60 min ahead of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Diamonds, membranes were preincubated for 60 min with xanomeline in the concentrations indicated on the x-axis and washed before addition of carbachol and $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. Curves are fits of Eq. 5 to the data. Data are means ± S.E.M of 4 independent experiments performed in quadruplicates.
Fig. 7

Stimulation of \([^{35}S]GTP\gamma S\) binding to \(G_i\), \(G_s\) and \(G_q\) \(G\)-proteins by carbachol and xanomeline.

\([^{35}S]GTP\gamma S\) binding (ordinate, fold increase over basal) to \(G_i\) (circles), \(G_s\) (squares), and \(G_q\) (diamonds) \(G\)-protein alpha subunits in CHO membranes expressing M1 (Left) or M2 (Right) receptors stimulated by carbachol (open symbols) or xanomeline (closed symbols) was determined using scintillation proximity assay. Upper row, agonists were added simultaneously with \([^{35}S]GTP\gamma S\). Middle row, agonists were added 60 min ahead of \([^{35}S]GTP\gamma S\). Lower row, membranes were preincubated with increasing concentrations of xanomeline as indicated on the x-axis and washed before incubation with \([^{35}S]GTP\gamma S\). Data are means ± S.E.M. of 3 to 6 independent experiments performed in quadruplicates.
**Tables**

Table 1

*Parameters of $[^{35}S]GTP\gamma S$ binding to membranes from CHO cells.*

Maximum stimulation ($E_{\text{MAX}}$) expressed as percent increase above basal binding and concentrations of agonists producing 50% of maximal response ($EC_{50}$) are expressed as negative logarithms of $[^{35}S]GTP\gamma S$ binding to membranes from CHO cells expressing M₁ or M₂ receptors, respectively. Data are means ± S.E.M. from 4 to 6 independent experiments performed in quadruplicates.
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<th>M&lt;sub&gt;2&lt;/sub&gt;</th>
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<td>E&lt;sub&gt;MAX&lt;/sub&gt; [% above basal]</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>E&lt;sub&gt;MAX&lt;/sub&gt; [% above basal]</td>
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<td>228 ± 9*</td>
<td>n.e.</td>
<td>n.e.</td>
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<td>6.08 ± 0.01</td>
<td>179 ± 7</td>
<td>6.12 ± 0.05</td>
<td>187 ± 0.06</td>
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<tr>
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<td>5.97 ± 0.03*</td>
<td>179 ± 8</td>
<td>n.m.</td>
<td>n.m.</td>
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<td>6.01 ± 0.05</td>
<td>186 ± 0.06</td>
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<td>184 ± 8</td>
<td>5.89 ± 0.03*</td>
<td>185 ± 0.09</td>
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<td>184 ± 9</td>
<td>5.63 ± 0.02*</td>
<td>182 ± 0.08</td>
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<td>n.m.</td>
<td>5.16 ± 0.05*</td>
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<td><strong>with 60 min preincubation</strong></td>
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<td>7.78 ± 0.03</td>
<td>75 ± 5*</td>
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<td>189 ± 6</td>
<td>6.12 ± 0.03</td>
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<td>n.m.</td>
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<td>189 ± 8</td>
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<td>4.91 ± 0.03*</td>
<td>194 ± 11</td>
<td>5.28 ± 0.03*</td>
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<tr>
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<td>n.m.</td>
<td>4.97 ± 0.04*</td>
<td>194 ± 7</td>
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<tr>
<td><strong>with 60 min preincubation followed by washing</strong></td>
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<tr>
<td>Xanomeline</td>
<td>5.98 ± 0.03</td>
<td>208 ± 6*</td>
<td>5.81 ± 0.03*</td>
<td>62 ± 5*</td>
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<td>5.84 ± 0.05*</td>
<td>194 ± 8</td>
<td>6.01 ± 0.02</td>
<td>173 ± 7*</td>
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<td>+ 10 µM xanomeline</td>
<td>5.42 ± 0.05*</td>
<td>178 ± 9</td>
<td>5.86 ± 0.01*</td>
<td>165 ± 7*</td>
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*p<0.05, significantly different from carbachol or carbachol alone by t-test or ANOVA followed by Dunnett’s test; n.e., no effect; n.m., not measured.
Table 2

Estimates of xanomeline $pK_B$ in antagonizing the responses to carbachol.

Negative logarithms of xanomeline equilibrium dissociation constant ($pK_B$) based on its effect on carbachol stimulated $[^{35}\text{S}]GTP\gamma S$ binding. Constants were obtained by non-linear 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 $[^3\text{H}]$NMS binding are shown for comparison.

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<th>$M_2$</th>
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<td>$pK_B$</td>
<td>$pEC_{50}$</td>
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<tr>
<td>Without</td>
<td>8.08 ± 0.04$^a$</td>
<td>8.04 ± 0.01$^a$</td>
</tr>
<tr>
<td>preincubation</td>
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<tr>
<td>60 min</td>
<td>8.15 ± 0.02$^b$</td>
<td>8.15 ± 0.04$^b$</td>
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<td>preincubation</td>
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<tr>
<td>60 min</td>
<td>5.69 ± 0.04$^{*a}$</td>
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<td>followed by</td>
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<td>washing</td>
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$^*, ^a$, significantly different from corresponding $pEC_{50}$ and $pK_I$, respectively; $^b$, significantly different from “without preincubation” by ANOVA followed by Tukey-Kramer post test; n.e., no effect.; n.m., not measured.
Table 3

Parameters of induced \[^{35}S\]GTP\(\gamma\)S 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 M1 or M2 receptors, respectively, was detected by scintillation proximity assay. Maximum stimulation (\(E_{MAX}\)) is expressed as percent increase above basal binding and concentrations of agonist producing 50% of maximal response (\(EC_{50}\)) is expressed as negative logarithms of \[^{35}S\]GTP\(\gamma\)S binding to \(G_i\), \(G_s\) and \(G_q\) G-proteins, respectively.

Data are means ± S.E.M. from 3 to 4 independent experiments performed in quadruplicates.
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<th>pEC_{50}</th>
<th>E_{MAX} [% above basal]</th>
<th>M_2</th>
<th>pEC_{50}</th>
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<tr>
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<td><strong>with 60 min preincubation</strong></td>
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<td>G_i</td>
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<td>6.39 ± 0.05</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>G_q</td>
<td>5.94 ± 0.06</td>
<td>289 ± 11</td>
<td>G_q</td>
<td>8.11 ± 0.04</td>
<td>283 ± 9</td>
</tr>
<tr>
<td><strong>xanomeline</strong></td>
<td></td>
<td></td>
<td><strong>carbachol</strong></td>
<td></td>
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</tr>
<tr>
<td>G_i</td>
<td>6.28 ± 0.06</td>
<td>68 ± 6</td>
<td>G_i</td>
<td>6.37 ± 0.04</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>G_s</td>
<td>5.94 ± 0.06</td>
<td>289 ± 11</td>
<td>G_s</td>
<td>6.39 ± 0.05</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>G_q</td>
<td>5.94 ± 0.06</td>
<td>289 ± 11</td>
<td>G_q</td>
<td>8.11 ± 0.04</td>
<td>283 ± 9</td>
</tr>
</tbody>
</table>

n.e., no effect; †,*, significantly different from “without preincubation” and “60 min preincubation, respectively (P<0.05, ANOVA followed by Tukey-Kramer post-test).
Figure 4

![Graphs M1 and M2 showing time-course of binding](image-url)