Mechanisms of M3 Muscarinic Receptor Regulation by Wash-Resistant Xanomeline Binding

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Key Words

Muscarinic receptors • Xanomeline • Chinese hamster ovary cells • Radioligand binding • Inositol phosphates • Allosteric modulation

Abstract

Background/Aims: Xanomeline has been shown to bind in a unique manner at M1 and M3 muscarinic receptors, with interactions at both the orthosteric site and an allosteric site. We have previously shown that brief exposure of Chinese hamster ovary cells that express the M3 receptor to xanomeline followed by removal of free agonist results in a delayed decrease in radioligand binding and receptor response to agonists. In the current study, we were interested in determining the mechanisms of this effect. **Methods:** Cells were treated with carbachol, pilocarpine or xanomeline for 1 h followed by washing and either used immediately or after waiting for 23 h. Control groups included cells that were not exposed to agonists and cells that were treated with agonists for 24 h. Radioligand binding and functional assays were conducted to determine the effects of agonist treatments. **Results:** The above treatment protocol with xanomeline resulted in similar effects of the binding of [³H]NMS and [³H]QNB. When receptor function is blocked using a variety of methods, the long-term effects of xanomeline binding were absent. **Conclusion:** Our data indicate that xanomeline

wash-resistant binding at the receptor allosteric site leads to receptor downregulation and that receptor activation is necessary for these effects.

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Introduction

There are five subtypes (M1–M5) of muscarinic receptors (mAChR) that are part of a large family of G-protein-coupled receptors. These receptors have seven transmembrane domains, in which interaction of the third cytoplasmic loop with the G-protein [1] and interactions within transmembrane domains 3, 5, 6, and 7 lead to receptor activation [2]. Each of the subtypes has a unique pattern of expression. M3 mAChR are widely expressed throughout the brain [2, 3]. In addition, they are also expressed in salivary glands, the gastrointestinal tract and other peripheral organs. They are coupled to G_q and G₁₁ G-proteins that in turn activate the phospholipase C signaling pathway [1]. Activation of this receptor subtype may be responsible for some of the adverse effects of cholinesterase inhibitors in Alzheimer's patients, such as salivation and gastrointestinal tract problems [4, 5].

mAChR have a high degree of homology across subtypes in the orthosteric binding domain, which has made it difficult to develop subtype-selective agonists [2, 6].

However, mAChR have been shown to have allosteric binding domains that may differ between subtypes, thus offering the potential for the development of such agents that would be of great therapeutic use [2]. Binding of a drug to an allosteric site on a receptor leads to changes in the conformation of the receptor's orthosteric domain that may result in receptor activation [7, 8]. Examples of such allosteric mAChR agonists include xanomeline [9], AC-42 [10] and WIN51708 [11].

Xanomeline has been proposed to bind in a unique manner at the M1 receptor. Binding occurs in a reversible manner at the orthosteric (primary) binding site and in a wash-resistant manner at an allosteric (secondary) binding site [12]. We have demonstrated that this unique binding profile also occurs at the M3 receptor [13]. At both the M1 and M3 receptors, wash-resistant xanomeline binding leads to a long-term decrease in cell-surface receptor expression and attenuation of receptor function [13, 14]. This is similar to the well-documented changes in receptor expression and sensitivity induced by conventional reversible muscarinic agonists [15–18]. The aim of the current study was to determine the mechanisms of the long-term effects of xanomeline binding at the M3 receptor.

Materials and Methods

Materials

[³H]N-methylscopolamine ([³H]NMS) (81 Ci/mmol) was purchased from DuPont (Wilmington, Del., USA); [³H]quinuclidinyl benzylate ([³H]QNB) (52 Ci/mmol) was purchased from DuPont; myo-[³H]inositol (71 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, UK); [¹⁴C]inositol-1-phosphate (300 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, Mo., USA); Dulbecco's modified Eagle's medium was purchased from Invitrogen (Carlsbad, Calif., USA); geneticin was obtained from Calbiochem (San Diego, Calif., USA); siRNA was purchased from Ambion (Austin, Tex., USA), and bovine calf serum was supplied by Hyclone Laboratories (Logan, Utah, USA). Xanomeline tartrate was a generous gift from Eli Lilly & Co. (Indianapolis, Ind., USA); all other reagents were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Cell Culture

Chinese hamster ovary (CHO) cells stably transfected with human M3 muscarinic acetylcholine receptor (hM3) (provided by Dr. M. Brann, University of Vermont Medical School) were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and 50 µg/ml geneticin. Cells were grown to confluency in 24-well plates or 10-cm dishes at 37°C in a humidified atmosphere consisting of 5% CO₂/95% air. All drug pretreatments and radioligand assays were performed on cells in monolayer unless otherwise noted.

Pretreatment with Agonist

CHO cells expressing hM3 receptor were incubated with agonist in monolayer at 37°C for 1 h. The cells were then washed 3 times with Hepes buffer (110 mmol/l NaCl, 5.4 mmol/l KCl, 1.8 mmol/l CaCl₂, 1 mmol/l MgSO₄, 25 mmol/l glucose, 20 mmol/l Hepes, 58 mmol/l sucrose; pH 7.4 ± 0.02; 340 ± 5 mosm) to remove any unbound drug. Cells were used immediately or after 23 h incubation in fresh culture medium. An additional group of cells was treated with agonist for 24 h prior to washing with Hepes buffer and used in the assay.

siRNA Treatment

CHO cells expressing hM3 receptors were incubated with 50 nmol/l siRNA each for G_q and G₁₁ G-proteins, with the transfection reagent Lipofectamine 2000, in Opti-Mem for 6–7 h. Media with no geneticin was added and the cells were allowed to incubate overnight. Subsequent pretreatment with xanomeline was the same as stated previously.

Inhibition of [³H]NMS Binding following Agonist Pretreatments

Two different protocols were used to assess agonist potency. CHO hM3 cells were incubated for 1 h concurrently with a fixed concentration of the muscarinic receptor ligand [³H]N-methylscopolamine ([³H]NMS) (0.2 nmol/l) and increasing concentrations of carbachol (100 nmol/l to 10 mmol/l) or pilocarpine (1 nmol/l to 100 µmol/l). In order to assess the effects of prolonged agonist treatments, cells were pretreated with increasing concentrations of carbachol or pilocarpine (1 pmol/l to 100 µmol/l) for 1 h followed by washing and used immediately or after incubation in ligand free media for 23 h. An additional group of cells was pretreated with agonists for 24 h prior to washing. In the binding assay, cells were incubated with [³H]NMS (0.2 nmol/l) for 1 h. In all instances, pretreatments and radioligand binding were performed in monolayer at 37°C. Non-specific binding was determined using 10 µmol/l atropine. Incubations were terminated by washing away free radioligand and then cells with bound radioligand were dissolved in 1 mol/l NaOH. Protein determinations were performed according to the method of Bradford [19]. Bound radioactivity (disintegrations per minute (dpm)) was quantitated by liquid scintillation spectrometry.

The second protocol was the same as above except saturating concentrations of the muscarinic receptor ligands [³H]NMS (2.9 nmol/l) or [³H]QNB (1.4 nmol/l) were used and cells were pretreated with xanomeline (1 nmol/l to 100 µmol/l). The concentrations of the [³H]NMS and [³H]QNB were chosen so that the percent of receptor occupancy would be the same.

Saturation Binding

Two different protocols were used to determine the total cell-surface receptor density and radioligand-receptor equilibrium dissociation constant. CHO hM3 cells were incubated in monolayer for 1 h at 37°C in the absence or in the presence of xanomeline (10 µmol/l) alone or in conjunction with atropine (10 µmol/l) followed by three washes with Hepes buffer and 23 h incubation in control media. A second group of cells was untreated or treated with xanomeline for 1 h, washed extensively and then treated with atropine for 23 h. Subsequently, cells were incubated in monolayer with increasing concentrations of [³H]NMS (0.01–6.5 nmol/l) for 1 h at 37°C. Non-specific binding was determined

using 10 $\mu\text{mol/l}$ atropine. Reactions were terminated as described above. Protein determinations were performed according to the method of Bradford [19]. Bound radioactivity (dpm) was quantitated by liquid scintillation spectrometry.

In the second protocol, siRNA transfections were performed as noted above. The transfection protocol was unsuccessful in cells seeded in 24-well plates due to significant cell death. We currently do not have an explanation for this phenomenon. Therefore, transfections were performed in 10-cm dishes. Subsequently, cells were incubated for 1 h at 37°C in the absence or presence of xanomeline (10 $\mu\text{mol/l}$) followed by washing and immediate use or further incubation for 23 h in control medium. Additional cells were treated with xanomeline for 24 h prior to washing. Cells were then removed from the 10-cm dishes by trypsinization and centrifuged (300 g, 2 min) and resuspended in Hepes buffer (3 times). Subsequently, cells were incubated with increasing concentrations of [^3H]NMS (0.04–8.5 nmol/l) in suspension for 1 h at 37°C using 10^5 cells/assay tube in a final volume of 1 ml. The reaction was terminated by filtration on Whatman GF/C filters (Whatman Schleicher & Schuell, Keene, N.H., USA) using a Brandel cell harvester (Brandel, Inc., Gaithersburg, Md., USA). Filters were washed 3 times with 4-ml aliquots of ice-cold saline and dried before radioactivity (dpm) was measured. Non-specific binding was determined using 10 $\mu\text{mol/l}$ atropine and protein content was determined using the method of Bradford [19].

Assay of Phosphoinositol (PI) Hydrolysis

Two different protocols were used to assess the functional response of the M3 receptor following exposure to various agonists. In all cases, cells in monolayer were loaded 24 h prior to use with *myo*-[^3H]inositol (1 $\mu\text{Ci/ml}$) at 37°C. In the first protocol, cells were pretreated for 1 h with carbachol (10 $\mu\text{mol/l}$) or pilocarpine (10 $\mu\text{mol/l}$), washed 3 times with Hepes buffer and used immediately or after 23 h incubation in control media. Additional groups of cells were pretreated with carbachol (10 $\mu\text{mol/l}$) or pilocarpine (10 $\mu\text{mol/l}$) for 24 h prior to washing 3 times with Hepes buffer. In the functional assay, cells were incubated in buffer with 10 mmol/l LiCl at 37°C for 1 h in the presence of carbachol (10 nmol/l to 10 mmol/l), pilocarpine (10 nmol/l to 1 mmol/l) or xanomeline (1 nmol/l to 100 $\mu\text{mol/l}$). The reaction was stopped with 0.3 mol/l HClO_4 and neutralized with 0.15 mol/l K_2CO_3 . The samples were centrifuged (1,500 g, 15 min) and total inositol phosphates were separated by ion-exchange chromatography (AG1-X8 resin). In all cases, [^{14}C]inositol-1-phosphate was used as an internal recovery standard. Radioactivity (dpm) was quantitated by liquid scintillation spectrometry.

The second protocol was the same as stated above with the following exceptions. First, only the prolonged xanomeline treatment groups (10 $\mu\text{mol/l}$; 1 h/wash/23 h wait) were used. Secondly, a receptor saturating concentration of atropine (10 $\mu\text{mol/l}$) was present concurrently with xanomeline during the initial pretreatment or during the 23-hour waiting period after washing away free xanomeline.

Data Analysis

All analyses were conducted using Prism 4.0 (GraphPad Software, Inc., San Diego, Calif., USA). Inhibition of [^3H]NMS binding isotherms was analyzed via non-linear regression to derive estimates of IC_{50} (midpoint location or potency parameter). Data were refitted according to both one- and two-site mass-action

binding models, and the better model was determined by an extra sum-of-squares test. The quasi-irreversible nature of xanomeline binding did not permit transforming IC_{50} values to inhibition constants because such transformation assumes reversible competitive interaction. Saturation binding isotherms were analyzed via non-linear regression to derive individual estimates of B_{max} (total cell-surface receptor density) and K_d (radioligand-receptor equilibrium dissociation constant). Even though radioligand depletion was minimal, all calculations were based on the concentration of free radioligand (added dpm minus bound dpm). PI hydrolysis isotherms were analyzed via non-linear regression. Data shown are the means $\pm$ SEM. Comparisons between mean values were performed by unpaired t tests, paired t tests or one-way analysis of variance (ANOVA), as appropriate. A probability (p) value <0.05 was taken to indicate statistical significance.

Results

Comparison of the Effects Resulting from Brief and Long-Term Xanomeline Treatment with Those of Carbachol and Pilocarpine

Previous research demonstrated that brief treatment of cells expressing the M3 receptor with xanomeline resulted in a monophasic inhibition curve of [^3H]NMS binding whereas long-term treatment produced a biphasic binding inhibition curve [13]. Experiments were designed to confirm that the observed effects of xanomeline are due to its unique interactions with the M3 receptor rather than a general property of agonists. Cells were exposed to increasing concentrations of the full agonist carbachol or the partial agonist pilocarpine [20] as described in Methods, and the specific binding of 0.2 nmol/l [^3H]NMS was determined. In untreated cells, carbachol and pilocarpine inhibited [^3H]NMS binding in a concentration-dependent manner when present concurrently with the radioligand, with complete inhibition of binding at high agonist concentrations ($98 \pm 0.6\%$ carbachol; $99 \pm 5.4\%$ pilocarpine). The binding isotherms of both agonists were best described by a single-site binding site and both exhibited a lower potency ($\text{pIC}_{50} = 3.6 \pm 0.05$ carbachol; 4.7 ± 0.11 pilocarpine) than xanomeline ($\text{pIC}_{50} = 7.1 \pm 0.1$) (fig. 1a and b, respectively [13]). When carbachol or pilocarpine were present for 1 h followed by washing and immediate use in the binding assay or incubation in control media for 23 h prior to the binding assay, no decrease in [^3H]NMS binding was observed (fig. 1a, b). This is in contrast to results to the wash-resistant effects of xanomeline that are potentiated by prolonged waiting after washing off free xanomeline [13]. However, when cells were incubated with carbachol or

Fig. 1. Inhibition of [3 H]NMS binding following acute and long-term treatment with agonists in CHO cells expressing the human M3 receptor. **a** Cells were pretreated with increasing concentrations of carbachol for 1 h followed by washing and used immediately ($\blacktriangle$) or after incubation for 23 h in control media ($\triangle$). Another group of cells was pretreated with carbachol for 24 h prior

to washing ($\diamond$). Subsequently, the specific binding of 0.2 nmol/l [3 H]NMS was determined following carbachol pretreatments or using naive cells in the continuous presence of carbachol ($\blacksquare$) 1 h at 37°C. **b** Cells received the same treatments as above (**a**), except with pilocarpine instead of carbachol. Values represent the means $\pm$ SE of 3 experiments conducted in triplicate.

Fig. 2. Effects of carbachol pretreatment on [3 H]NMS saturation binding in CHO cells expressing human M3 receptors. Cells were pretreated with 10 μ mol/l carbachol for 1 h followed by washing and incubation in the absence of free carbachol for 23 h ($\triangle$) or treated with carbachol for 24 h prior to washing ($\diamond$). Subsequently, untreated cells ($\blacksquare$) or pretreated cells were incubated with increasing concentrations of [3 H]NMS (0.01–7 nmol/l) for 1 h at 37°C. Non-specific binding was determined in the presence of 10 μ mol/l atropine. Values represent the means $\pm$ SE of 3 experiments conducted in triplicate.

pilocarpine for 24 h followed by washing, there was a concentration-dependent inhibition of [3 H]NMS binding that was best fit by a single-site binding isotherm with potencies of $pCI_{50} = 7.8 \pm 0.19$ for carbachol and 6.7 ± 0.05 for pilocarpine, with a maximal inhibition of [3 H]NMS binding of 48 ± 1.3 and $40 \pm 5.4\%$, respectively (fig. 1a and b, respectively). Once again, this is in contrast to the effects of xanomeline under similar conditions whereby inhibition of [3 H]NMS binding results in a biphasic curve and reaches a maximum of nearly 100% [13]. Previous research demonstrated a decrease in protein content following xanomeline pretreatments at the M1 receptor [14]. Thus, experiments were undertaken to determine if there were similar alterations in protein content following the various carbachol or pilocarpine pretreatments described above at the M3 receptor. Measurement of protein content using the method of Bradford [19] did not detect any differences between the aforementioned treatment and control groups (data not shown).

Effects of Long-Term Exposure of Cells to Carbachol on [3 H]NMS Saturation Binding

Previous research has shown that prolonged exposure of cells expressing muscarinic receptors to carbachol leads to a decrease in cell-surface receptor expression with no change in radioligand affinity [16–18, 21–23]. To

Table 1. Changes in carbachol, pilocarpine or xanomeline stimulation of PI hydrolysis in M3-CHO cells following pretreatment with reversible agonists. Cells were pretreated with 10 $\mu\text{mol/l}$ carbachol or pilocarpine for 1 h followed by washing and incubation for 23 h in the absence of free agonist. Additional groups of cells were treated with 10 $\mu\text{mol/l}$ carbachol or pilocarpine for 24 h prior to washing. In the functional assay, cells were stimulated with increasing concentrations of carbachol, pilocarpine or xanomeline for 1 h at 37°C in the presence of 10 mmol/l LiCl. Parameters ($\pm$ SEM) were derived from computer-assisted non-linear regression analysis

Pretreatment group	Carbachol stimulation			Pilocarpine stimulation			Xanomeline stimulation		
	pEC ₅₀ ^a	E _{max} ^b	n ^c	pEC ₅₀	E _{max}	n	pEC ₅₀	E _{max}	n
<i>Carbachol</i>									
Control (untreated)	6.1 $\pm$ 0.11	25,400 $\pm$ 2,400	6				5.7 $\pm$ 0.35	21,300 $\pm$ 3,300	3
1 h/washout/23 h wait	6.0 $\pm$ 0.43	25,600 $\pm$ 3,300	3						
24 h/washout/no wait	4.7 $\pm$ 0.27 ¹	13,200 $\pm$ 3,200 ¹	3				ND	ND	3
<i>Pilocarpine</i>									
Control (untreated)	6.1 $\pm$ 0.11	25,400 $\pm$ 2,400	6	5.8 $\pm$ 0.04	19,000 $\pm$ 1,900	3			
1 h/washout/23 h wait	6.2 $\pm$ 0.08	26,300 $\pm$ 3,400	3	5.9 $\pm$ 0.03	16,400 $\pm$ 1,600	3			
24 h/washout/no wait	5.8 $\pm$ 0.11	30,500 $\pm$ 2,300	3	5.3 $\pm$ 0.11 ²	10,800 $\pm$ 3,200	3			

ND = Parameter value was not determined.

^a Negative logarithm of the midpoint (potency) parameter.

^b Maximal response. Values are expressed as dpm/well after correcting for standard recovery.

^c Number of experiments.

¹ ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC₅₀ or E_{max} where indicated between carbachol-pretreated groups with carbachol stimulation compared to carbachol stimulation in untreated cells.

² ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC₅₀ or E_{max} where indicated between pilocarpine-pretreated groups with pilocarpine stimulation compared to pilocarpine stimulation in untreated cells.

confirm these results in our experimental paradigm, we determined the effects of long-term exposure of M3 receptors to this agonist. CHO cells expressing the M3 receptor were subjected to the various pretreatments with 10 $\mu\text{mol/l}$ carbachol as detailed above, washed, then exposed to increasing concentrations of [³H]NMS. When cells were treated with carbachol for 1 h followed by washing and incubation in control media for 23 h, there was no change in the maximal binding ($B_{\text{max}} = 56,600 \pm 4,200$ dpm/well) or radioligand affinity ($K_d = 0.28 \pm 0.01$) compared to control ($B_{\text{max}} = 65,000 \pm 2,900$ dpm/well; $K_d = 0.34 \pm 0.01$) (fig. 2). In contrast, pretreatment with xanomeline under the same conditions resulted in a decrease in cell-surface receptor density [13]. In agreement with previous literature [16–18, 23], pretreatment with carbachol for 24 h resulted in a significant ($p < 0.05$) decrease in maximal binding ($B_{\text{max}} = 26,200 \pm 2,700$ dpm/well). However, there was an unexpected significant ($p < 0.05$) increase in radioligand affinity ($K_d = 0.23 \pm 0.03$) compared to control (fig. 2). This is in stark contrast to the marked decrease in radioligand affinity observed following pretreatment with xanomeline for 24 h [13].

Effects of Long-Term Carbachol or Pilocarpine Exposure on the Ability of Agonists to Stimulate Inositol Phosphate Production

Pretreatment of cells with xanomeline either acutely or for long term resulted in antagonism of the functional response to subsequent stimulation with agonists [13]. Thus, experiments were conducted to ascertain if the observed delayed effects of wash-resistant xanomeline binding on receptor function were unique to xanomeline or are due to general effects elicited by long-term exposure to all muscarinic agonists. A 10- $\mu\text{mol/l}$ concentration of either carbachol or pilocarpine was selected, since at this concentration both agonists produce maximal receptor activation and inhibition of [³H]NMS binding following prolonged exposure.

When cells were pretreated with carbachol or pilocarpine for 1 h followed by washing and incubation in control media for 23 h, no change in efficacy or potency of carbachol in stimulating phosphoinositide hydrolysis was observed (fig. 3a, b; table 1). In contrast, pretreatment with carbachol for 24 h followed by washing resulted in a significant ($p < 0.05$) decrease in the maximal response and potency of a second addition of carbachol (fig. 3a;

Fig. 3. Effects of long-term agonist exposure on the ability of various muscarinic agonists to stimulate inositol phosphates production. **a** Cells were pretreated with 10 $\mu\text{mol/l}$ carbachol for 1 h followed by washing and waiting 23 h in control media ($\square$) or treated for 24 h with carbachol prior to washing ($\diamond$). Subsequently, treated or untreated ($\blacksquare$) cells were stimulated with increasing concentrations of carbachol and the accumulation of inositol phosphates was

determined in the presence of 10 mmol/l LiCl over a 1 h incubation period at 37°C. **b** Cells were treated with pilocarpine similar to above (**a**), then stimulated with carbachol 10 $\mu\text{mol/l}$. **c** Cells were treated with pilocarpine as above (**a**), then stimulated with pilocarpine. **d** Cells were treated with carbachol similar to above (**a**), then stimulated with xanomeline. Values represent the means $\pm$ SE of 3–6 experiments conducted in triplicate.

table 1). A similar long-term treatment with pilocarpine did not alter either concentration-response parameters of carbachol (fig. 3b; table 1). In addition, pretreatment with pilocarpine for 1 h followed by washing and waiting did not modify the concentration-response relationship of pilocarpine. However, pilocarpine treatment for 24 h prior to washing followed by a second addition of pilocarpine demonstrated significantly ($p < 0.05$) decreased potency as well as marked decrease in efficacy (fig. 3c), similar to what was observed with carbachol pretreatment followed by carbachol stimulation (fig. 3a).

Further experiments were performed to determine the effects of 24-hour carbachol pretreatment on the ability of xanomeline to stimulate a response (fig. 3d). Pretreat-

ment of cells for 24 h with carbachol followed by washing and stimulation with increasing concentrations of xanomeline resulted in minimal production of inositol phosphates observed only at the highest concentration of xanomeline used (100 $\mu\text{mol/l}$) (fig. 3d).

Comparison of the Effects of Brief and Long-Term Exposure of Cells to Xanomeline on [^3H]QNB and [^3H]NMS Binding

Prolonged agonist exposure can lead to receptor internalization, downregulation or uncoupling of the receptor from its G-protein [24, 25]. The current set of experiments was conducted to differentiate the long-term effects of xanomeline on internalization and downregulation of the

Fig. 4. Comparison of the effects of xanomeline treatment on the specific binding of [3 H]QNB and [3 H]NMS in CHO cells expressing human M3 receptors. **a** Cells were pretreated with increasing concentrations of xanomeline for 1 h, washed and used immediately ($\blacktriangle$) or after incubation in control medium for 23 h ($\triangle$). An additional group of cells was pretreated with xanomeline for 24 h

prior to washing ($\blacklozenge$). The binding of 1.4 nmol/l [3 H]QNB was determined in untreated cells in the concurrent presence of xanomeline ($\blacksquare$) or following various xanomeline pretreatments. **b** Cells received the same treatments as above (**a**), except that the binding assay was conducted with 2.9 nmol/l [3 H]NMS. Values represent the means $\pm$ SE of 3–4 experiments conducted in triplicate.

M3 receptor. QNB is a lipophilic muscarinic receptor antagonist that is able to bind to both cell-surface and internalized (but intact) receptors [26, 27]. In contrast, NMS (a permanently-charged quaternary amine) is only able to bind to receptors expressed on the cell surface [28]. We compared effects of the various xanomeline treatment paradigms on the specific binding of both radioligands. Receptor-saturating concentrations of [3 H]QNB and [3 H]NMS were used to confine effects of xanomeline to alterations in the number of receptors without interference from changes in radioligand affinity.

Cells were exposed to increasing concentrations of xanomeline for the various treatment conditions and the binding of either 1.4 nmol/l [3 H]QNB or 2.8 nmol/l [3 H]NMS was determined. These concentrations were chosen because they result in similar receptor occupancy by either ligand (approximately 93% of total receptors). In untreated cells, inclusion of xanomeline in the binding assay resulted in complete concentration-dependent inhibition of binding of both radioligands with similar potency (fig. 4a, b; table 2). Cells pretreated with xanomeline for 1 h followed by washing and immediate use in the binding assay showed wash-resistant xanomeline binding that exhibited a lower potency against either radioligand compared to when xanomeline was included in the binding assay ($p < 0.05$) (fig. 4a, b; table 2). Additionally, inhibition of [3 H]NMS binding was incomplete following this treatment. When cells were pretreated with xanome-

line for 1 h followed by washing and waiting 23 h, there was a significant ($p < 0.05$) increase in the apparent potency of xanomeline as compared to its acute wash-resistant effects. Surprisingly, this increase was greater for NMS compared to QNB binding. Noteworthy, inhibition of binding under these treatment conditions was incomplete for both radioligands resulting in a maximal inhibition of approximately 35–45% (fig. 4a, b; table 2).

An additional group of cells was incubated with increasing concentrations of xanomeline for 24 h followed by washing and immediate use in radioligand binding assays. This condition resulted in biphasic xanomeline inhibition curves of either radioligand, with the high-potency site resulting in approximately 42–43% of the binding sites for both radioligands. The apparent high-potency site under these conditions demonstrated a markedly higher potency than that observed in the case of cells that were briefly treated with xanomeline, washed and used immediately or further incubated in control media for a day (table 2). Once again the inhibition of binding was incomplete for both radioligands under these conditions (table 2).

Effects of Atropine on Long-Term Xanomeline-Induced Inhibition of [3 H]NMS Binding

Experiments were designed to determine the role of xanomeline interaction with the receptor's orthosteric domain in the development of its long-term effects. A re-

Table 2. Inhibition of radioligand binding using a receptor-saturating concentration of [³H]QNB or [³H]NMS following various 1-hour xanomeline pretreatment and waiting periods in M3-CHO cells. Cells were treated with increasing concentrations of xanomeline for 1 h followed by washing and immediate use in the binding assay or subsequent to incubation for 23 h in the absence of free xanomeline. An additional group of cells was treated with xanomeline for 24 h prior to washing. Cells were then incubated with 1.4 nmol/l [³H]QNB or 2.9 nmol/l [³H]NMS for 1 h at 37°C. Parameters (±SEM) were derived from computer-assisted non-linear regression analysis

Pretreatment group	[³ H]QNB					[³ H]NMS				
	pIC ₅₀ ^a	pIC ₅₀ high ^b	pIC ₅₀ low ^c	I _{max} ^d	n ^e	pIC ₅₀	pIC ₅₀ high	pIC ₅₀ low	I _{max}	n
Control (untreated) ^f	6.4 ± 0.11			104 ± 0.5	3	6.6 ± 0.16			97 ± 1.2	3
1 h/washout/no wait	4.9 ± 0.12 ¹			88 ± 2.6	3	5.0 ± 0.09 ¹			77 ± 1.9 ²	3
1 h/washout/23 h wait	6.7 ± 0.18 ³			34 ± 8.0 ²	3	7.9 ± 0.30 ⁴			43 ± 1.1 ²	4
24 h/washout/no wait		8.8 ± 0.13 ³ (43 ± 3.3%)	5.1 ± 0.01	80 ± 3.2 ²	3		9.2 ± 0.47 ⁴ (42 ± 3.3%)	6.0 ± 0.37	70 ± 1.5 ²	4

^a Negative logarithm of the IC₅₀ for binding to a single potency site.

^b Negative logarithm of the IC₅₀ for the high-potency agonist binding site; percent of binding sites shown in parentheses.

^c Negative logarithm of the IC₅₀ for the low-potency agonist binding site.

^d Maximal percent inhibition of radioligand binding.

^e Number of experiments.

^f Control cells were incubated simultaneously with xanomeline and the radioligand.

¹ Unpaired t test detected a significant difference (p < 0.05) in pIC₅₀ of QNB or NMS binding between the 1 h/wash/no wait group compared to their respective controls.

² Significantly different from 100 as determined by ANOVA followed by Dunnett's post-test comparison.

³ ANOVA followed by Dunnett's post-test comparison detected a significant difference (p < 0.05) in pIC₅₀ for QNB binding where indicated between the 1 h/wash/23 h wait or 24 h/wash/no wait pretreated groups compared to 1 h/wash/no wait treatment.

⁴ ANOVA followed by Dunnett's post-test comparison detected a significant difference (p < 0.05) in pIC₅₀ for NMS binding where indicated between the 1 h/wash/23 h wait or 24 h/wash/no wait groups compared to 1 h/wash/no wait treatment.

ceptor-saturating concentration of atropine (10 μmol/l), a muscarinic receptor antagonist, was added either concurrently with 10 μmol/l xanomeline pretreatment or during the waiting period following xanomeline washout. [³H]NMS saturation binding isotherms were constructed to examine the effects of blocking the orthosteric site with atropine on xanomeline-induced changes in both radioligand affinity and maximal binding. Since atropine has been shown to induce its own effects [23, 29], comparisons were made between cells treated with both atropine and xanomeline and those treated with atropine alone. The simultaneous presence of atropine during incubation of cells with xanomeline for 1 h followed by washing and waiting 23 h in control medium did not influence the significant (p < 0.05) decrease in [³H]NMS maximal binding induced by xanomeline pretreatment alone (fig. 5a, b; table 3). However, when atropine was present during the 23-hour incubation following xanomeline pretreatment and washing, the long-term changes in radioligand maximal binding were no longer evident (fig. 5c; table 3). In contrast, either protocol of atropine

treatment abolished the long-term effects of xanomeline on radioligand affinity. All treatment groups had similar protein content.

Effects of Blocking Receptor Function Using siRNA against G_q and G₁₁ G-Proteins

Experiments were conducted to further examine the role of receptor activation on the long-term effects of xanomeline treatments on [³H]NMS saturation binding parameters using another experimental approach. It is known that the M3 receptor exerts its functional response via activation of the G_{q/11} class of G-proteins [1]. Thus, we utilized siRNA technology to block expression of these G-proteins. Cells were transfected with 50 μmol/l siRNA targeting G_q and G₁₁ G-proteins in an effort to block receptor function. Western blot analysis confirmed that transfection with siRNA targeting G_{q/11} resulted in a nearly 100% decrease in the detectable expression of G_{q/11} class of G-proteins (fig. 6a). In addition, loss of receptor function following transfection was assessed by determining agonist-mediated production of inositol phos-

phates using two concentrations of carbachol (1 and 100 μ mol/l) or xanomeline (100 nmol/l and 10 μ mol/l). The response to carbachol was decreased by 62 and 27%, respectively, and the response to xanomeline was reduced by 78 and 82%, respectively (fig. 6b). The ability of agonists to stimulate a functional response subsequent to siRNA treatment suggests that although the levels of $G_{q/11}$ G-proteins were no longer detectable following siRNA treatment, some $G_{q/11}$ G-proteins were still present. A [3 H]NMS saturation binding paradigm was applied to determine the effects of blocking receptor function on xanomeline-induced changes in both radioligand affinity and maximal binding. However, while attached cells were pretreated with xanomeline, binding experiments were conducted in suspension due to technical limitations (see Methods). In control non-siRNA-transfected cells, a trend toward a decrease in radioligand affinity was observed following 1-hour immediate-use xanome-

line pretreatment as well as pretreatment with xanomeline for 24 h prior to washing. Additionally, a trend toward a decrease in maximal binding resulted with 1-hour xanomeline pretreatment followed by washing and waiting 23 h or treatment for 24 h with xanomeline followed by washing and no waiting. These effects on radioligand affinity and maximal binding were similar, although less pronounced, than those obtained from [3 H]NMS saturation binding performed in monolayer [13] (fig. 6c; table 4). This is similar to previous work by Thompson and Fisher [30] in which there was a greater loss of receptors in cells that were pretreated with carbachol in monolayer compared to cells pretreated in suspension prior to the radioligand binding assay. Cells transfected with siRNA displayed a trend toward a decrease in radioligand affinity for all xanomeline pretreatment groups except the 24-hour treatment group that had a significant ($p < 0.05$) decrease in radioligand affinity compared to control.

Table 3. Effects of xanomeline pretreatment and washout in the absence or presence of atropine on [³H]NMS saturation binding and functional parameters of carbachol stimulation in M3-CHO cells. Cells were pretreated for 1 h with 10 μmol/l xanomeline in the absence or presence of 10 μmol/l atropine followed by washing and waiting for 23 h in the absence or presence of atropine. In the binding assay, cells were then incubated with increasing concentrations of [³H]NMS (0.02–13.5 nmol/l) for 1 h at 37°C. In the functional assay, cells were stimulated with increasing concentrations of carbachol for 1 h at 37°C in the presence of 10 mmol/l LiCl. Parameters (±SEM) were derived from computer-assisted non-linear regression analysis

Pretreatment group	Saturation binding parameters			Functional parameters		
	K _d , nmol/l ^a	B _{max} ^b	n ^c	pEC ₅₀ ^d	E _{max} ^e	n
Control (untreated) ^f	0.38 ± 0.05	66,000 ± 11,000	4	6.0 ± 0.18	29,600 ± 2,700	3
1 h xanomeline/washout/23 h wait	0.47 ± 0.03 ¹	27,000 ± 5,500 ¹	4	5.3 ± 0.07 ¹	26,100 ± 2,300	3
1 h atropine/washout/23 h wait	0.58 ± 0.05	67,000 ± 7,400	4	5.6 ± 0.17	24,200 ± 2,000	3
1 h xanomeline and atropine/washout/23 h wait	0.66 ± 0.07	40,000 ± 7,400 ²	4	5.3 ± 0.16	24,600 ± 1,600	3
23 h atropine/washout/no wait	3.20 ± 0.39	68,000 ± 11,800	4	4.2 ± 0.01	28,200 ± 3,200	3
1 h xanomeline/washout/23 h atropine/washout/no wait	3.25 ± 0.71	67,000 ± 17,000	4	4.2 ± 0.09	21,100 ± 830	3

No significant difference was detected in K_d, B_{max}, pEC₅₀ or E_{max} between 1 h xanomeline/wash/23 h atropine/wash/no wait compared to 23 h atropine/wash/no wait.

^a Equilibrium dissociation constant for [³H]NMS binding.

^b Maximal cell-surface receptor density (dpm/well).

^c Number of experiments.

^d Negative logarithm of midpoint (potency) parameter.

^e Maximal response. Values are expressed as dpm/well after correcting for standard recovery.

^f Control experiments were conducted on untreated cells in the binding assay and stimulated with carbachol in the functional assay.

¹ Paired t test detected a significant difference (p < 0.05) in K_d, B_{max} or pEC₅₀ between the 1 h/wash/23 h wait xanomeline-pretreated group compared to control. No significant difference was detected in E_{max}.

² Paired t test detected a significant difference (p < 0.05) in B_{max} between the 1 h xanomeline and atropine/wash/23 h wait-pretreated group compared to 1 h atropine/wash/23 h wait treatment. No significant difference was detected in K_d, pEC₅₀, or E_{max}.

Table 4. Effects of obliterating receptor function using siRNA against G_q and G₁₁ G-proteins on saturation binding parameters in M3-CHO cells in suspension. Cells were untreated or treated with 50 nmol/l siRNA for G_q and G₁₁ G-proteins. Subsequently, cells were treated with 10 μmol/l xanomeline for 1 h followed by washing and used immediately or after 23 h incubation in control media. An additional group of cells was treated with xanomeline for 24 h prior to washing. Cells were then incubated in suspension with increasing concentrations of [³H]NMS (0.04–7.4 nmol/l) at 37°C for 1 h. Parameters (±SEM) were derived from computer-assisted non-linear regression analysis. All other details as for table 2

Xanomeline pretreatment group	Control (no siRNA)			siRNA		
	K _d , nmol/l	B _{max}	n	K _d , nmol/l	B _{max}	n
Control (untreated)	0.22 ± 0.05	3,500 ± 500	3	0.22 ± 0.06	3,400 ± 300	3
1 h/washout/no wait	0.33 ± 0.04	3,800 ± 400	3	0.40 ± 0.07	4,400 ± 300	3
1 h/washout/23 h wait	0.24 ± 0.02	2,600 ± 100	3	0.36 ± 0.01	3,200 ± 200	3
24 h/washout/no wait	0.48 ± 0.12	2,600 ± 300	3	0.63 ± 0.13 ¹	3,700 ± 500	3

¹ ANOVA followed by Dunnett's post-test detected a significant difference (p < 0.05) in K_d where indicated between the siRNA-xanomeline-pretreated groups compared with siRNA-treated control.

Fig. 6. Effects of blocking receptor function using siRNA targeted against G_q and G₁₁ G-proteins on long-term xanomeline-induced inhibition of [³H]NMS binding. **a** Cells were left untreated (lanes 1 and 3) or treated with 50 nmol/l siRNA for G_q and G₁₁ G-proteins (lanes 2 and 4). The level of G_{q/11} G-protein expression was determined by Western blot. **b** Cells were untreated (open bars) or treated with 50 nmol/l siRNA for G_q and G₁₁ G-proteins (solid bars). Inositol phosphate production was determined following stimulation with two concentrations of carbachol (1 or 100 μmol/l) or xanomeline (100 nmol/l or 10 μmol/l). **c** Cells were

treated with xanomeline (10 μmol/l) for 1 h followed by washing and used immediately (▲) or after 23 h incubation in control medium (△). An additional group of cells was treated with xanomeline for 24 h prior to washing (◆). Subsequently, untreated cells (■) or pretreated cells were incubated in suspension with increasing concentrations of [³H]NMS (0.01–7.5 nmol/l) for 1 h at 37°C. **d** Cells were treated with 50 nmol/l siRNA for G_q and G₁₁ G-proteins to inhibit receptor function and then subjected to various xanomeline treatment protocols as above (**c**). Values represent the means ± SE of 3 experiments conducted in triplicate.

There was no change in maximal binding for any of the siRNA-treated groups compared to control (fig. 6d; table 4). Protein content remained the same across the various pretreatment groups (data not shown).

Effects of Atropine on Xanomeline-Induced Long-Term Alterations in Receptor Sensitivity

Similar to the previously described [³H]NMS binding experiments, functional experiments were conducted to determine the effects of blocking the primary binding site with a receptor-saturating concentration of atropine

(10 μmol/l), either concurrently with 10 μmol/l xanomeline pretreatment for 1 h or during the 23-hour waiting period following xanomeline pretreatment and washing on the ability of carbachol to stimulate the production of inositol phosphates. In contrast to results obtained from binding experiments, simultaneous presence of atropine with xanomeline for 1 h followed by washing and waiting for 23 h in control medium abolished the effects of xanomeline on carbachol potency (fig. 7a, b; table 3). The presence of atropine during the 23-hour incubation following xanomeline treatment and washout also eliminat-

Fig. 7. Effects of atropine on long-term modulation of inositol phosphates production by xanomeline in CHO cells expressing human M3 receptors. **a** Cells were either untreated (■) or treated with 10 $\mu\text{mol/l}$ xanomeline for 1 h followed by washing and waiting for 23 h in control medium (△). **b** Cells were treated with atropine (10 $\mu\text{mol/l}$) for 1 h (●) or treated simultaneously with atropine and xanomeline for 1 h (○) followed by washing and waiting 23 h in control medium. **c** Cells were untreated (◆) or treated with xanomeline for 1 h (◇) followed by washing and incubation with atropine for 23 h prior to washing. In all cases, the production of inositol phosphates was determined after the various pre-treatments in the presence of increasing concentrations of carbachol for 1 h at 37°C in the presence of 10 mmol/l LiCl. Values represent the means $\pm$ SE of 3 experiments conducted in triplicate.

ed xanomeline-induced changes in carbachol potency, although a decrease in efficacy was observed compared to control (fig. 7c; table 3).

Differential Role of Wash-Resistant Binding versus Persistent Receptor Activation in Xanomeline Long-Term Effects on Saturation Binding

Shortening the hydrophobic chain of xanomeline has previously been shown to result in diminishing its wash-resistant binding and functional properties at the M1 receptor [31]. Thus, the propyl analog of xanomeline (three carbons shorter than xanomeline) does not display wash-resistant binding or stimulate a functional response at the M1 receptor. At the M3 receptor, this analog exhibited wash-resistant binding, albeit with very low potency compared to xanomeline (data not shown). While this analog produced 45% of the PI response to xanomeline when present in the assay of receptor function, it did not

display any wash-resistant receptor activation (data not shown). We therefore utilized the propyl analog to discern the role of persistent receptor activation, versus wash-resistant binding per se in the observed long-term effects of xanomeline at the M3 receptor. When cells were exposed to the propyl analog at 100 $\mu\text{mol/l}$ for 1 h followed by washing and overnight incubation in control media, there was only a minute decrease in cell-surface receptor density (B_{max} 51,500 $\pm$ 3,100 dpm/well) in conjunction with a slight increase in radioligand affinity (K_d 0.74 $\pm$ 0.01 nmol/l) compared to control (B_{max} 73,400 $\pm$ 4,900 dpm/well; K_d 0.74 $\pm$ 0.01) (fig. 8). In contrast, 24-hour exposure of cells to the propyl analog prior to washing resulted in an anomalous significant ($p < 0.05$) increase in cell-surface receptor density (133,100 $\pm$ 10,500 dpm/well) along with a significant ($p < 0.05$) increase in the K_d of [^3H]NMS to 5.73 $\pm$ 0.74 nmol/l compared to control (fig. 8).

Fig. 8. Effects of wash-resistant binding in the absence of receptor activation on the ability of the propyl analog of xanomeline to initiate long-term effects on saturation binding parameters in CHO cells expressing human M3 receptors. Cells were pretreated with 100 $\mu\text{mol/l}$ of the propyl xanomeline analog for 1 h followed by washing and incubation in the absence of free drug for 23 h (Δ). An additional group of cells was treated for 24 h with the propyl analog prior to washing ($\blacklozenge$). Subsequently, untreated cells ($\blacksquare$) or pretreated cells were incubated with increasing concentrations of [^3H]NMS (0.02–15 nmol/l) for 1 h at 37°C. Non-specific binding was determined in the presence of 10 $\mu\text{mol/l}$ atropine. Values represent the means $\pm$ SE of 3 experiments conducted in duplicate.

Fig. 9. Effects of blocking receptor activation on the ability of xanomeline to initiate long-term effects on [^3H]NMS saturation binding parameters in CHO cells expressing human M3 receptors. Cells were pretreated with 10 $\mu\text{mol/l}$ xanomeline for 1 h at 37°C followed by washing and incubation in the absence of free xanomeline for 23 h at 4°C (Δ). Additional groups of cells were treated with 10 $\mu\text{mol/l}$ ($\blacklozenge$) or 100 $\mu\text{mol/l}$ ($\diamond$) xanomeline for 24 h at 4°C prior to washing. Subsequently, untreated cells incubated at 4°C ($\blacksquare$) or pretreated cells were incubated with increasing concentrations of [^3H]NMS (0.02–16.5 nmol/l) for 1 h at 37°C. Non-specific binding was determined in the presence of 10 $\mu\text{mol/l}$ atropine. Values represent the means $\pm$ SE of 3–4 experiments conducted in duplicate.

Effects of Wash-Resistant Binding in the Absence of Receptor Activation on the Ability of Xanomeline to Mediate Long-Term Effects on Saturation Binding

Previous literature has shown that wash-resistant inhibition of [^3H]NMS binding by xanomeline occurs with markedly lower potency at 4°C than at 37°C [31]. However, functional assays demonstrated that no functional response was elicited by xanomeline at 4°C (data not shown). Therefore, experiments were conducted at 4°C to further demonstrate the need for a functional receptor to observe long-term effects of xanomeline. Increasing concentrations of [^3H]NMS were used to determine cell-surface receptor density and radioligand affinity. Exposure of cells to xanomeline for 1 h at 37°C followed by washing and 23-hour incubation at 4°C in the absence of free xanomeline did not result in changes in maximal cell-surface receptor density ($33,400 \pm 3,600$ dpm/well) or radioligand affinity (K_d 0.34 ± 0.03 nmol/l) compared to the 4°C control (B_{max} $35,000 \pm 2,800$; K_d 0.24 ± 0.004 nmol/l) (fig. 9). However, when cells were exposed to xanomeline at 4°C for 24 h prior to washing

there was a significant ($p < 0.05$) concentration-dependent increase in [^3H]NMS K_d (1.08 ± 0.06 and 6.25 ± 0.44 nmol/l, respectively) in the absence of a change in cell-surface receptor density ($35,300 \pm 4,100$ and $32,500 \pm 2,000$, respectively) compared to the 4°C control (fig. 9).

Discussion

In the literature, xanomeline has been proposed to be a functionally selective M1/M4 muscarinic agonist [4, 32]. In agreement with previous findings, we have demonstrated that xanomeline acts as a partial agonist at the M3 receptor [4, 13]. Inadvertent activation of the M3 receptor by xanomeline may account for some of the side effects that were seen in clinical trials such as gastrointestinal tract problems and hypersalivation [4, 5]. In addition, we have demonstrated that long-term changes in M3 receptor binding and function are evident following acute pretreatment with xanomeline [13]. The current

study was undertaken to discern the mechanisms underlying long-term regulation of the M3 receptor by xanomeline.

Pretreatment with pilocarpine or carbachol followed by washing did not result in acute or delayed effects on inhibition of [3 H]NMS binding, suggesting that the wash-resistant effects of xanomeline are ligand-specific rather than a general property of brief agonist binding to the receptor (fig. 1). Exposure of cells to either carbachol or pilocarpine for 24 h prior to washing resulted in a monophasic concentration-dependent inhibition of [3 H]NMS binding that exhibited markedly higher agonist potencies than those obtained when the agonists were added directly to untreated cells in the binding assay medium. In contrast, treatment with xanomeline for 24 h prior to washing resulted in a biphasic binding curve [13]. While the profile of xanomeline treatment is very different from that of other agonists, the high-potency component of inhibition of [3 H]NMS binding following xanomeline treatment resembles that of the monophasic attenuation of binding caused by 24-hour pretreatment with other agonists. This similarity suggests that the former might be the result of a common receptor response to long-term activation. In support of this notion, incubation of cells with carbachol or pilocarpine for 24 h prior to washing resulted in incomplete inhibition of [3 H]NMS binding that was similar in magnitude to the proportion of high-potency binding induced by long-term incubation of xanomeline-pretreated and washed cells [13]. This might be due to an inherent limited susceptibility of the receptor to agonist-induced regulation. In contrast, 24-hour pretreatment with higher concentrations of xanomeline resulted in complete inhibition of [3 H]NMS binding [13], further suggesting that xanomeline regulates the receptor in a distinct manner compared to other conventional agonists.

The specific effects of wash-resistant xanomeline binding were also observed in [3 H]NMS saturation binding experiments. Exposure of cells to carbachol for 1 h followed by washing and incubation in control media for 23 h resulted in radioligand binding parameters that were not different from control (fig. 2). However, pretreatment with xanomeline under the same conditions led to a decrease in cell-surface receptor density [13]. This suggests that long-term receptor regulation by xanomeline is the result of its persistent binding to the receptor rather than the initial interaction with the receptor prior to washing. Similarly to previously published results [16–18, 23], exposure of cells to carbachol for 24 h prior to washing resulted in a decrease in receptor density. However, an in-

crease in radioligand affinity was also observed, which is in contrast to previous findings. At present we do not have a reasonable explanation for this anomalous observation. Pretreatment with xanomeline for 24 h prior to washing also resulted in a decrease in cell-surface receptor expression, but this effect was accompanied by a significant decrease in radioligand affinity [13]. These observations may be explained by interference of wash-resistant xanomeline with [3 H]NMS binding in an allosteric manner.

The observed delayed effects of acute xanomeline wash-resistant binding were associated with attenuation of the receptor response to activation by carbachol. Control experiments with the conventional agonists carbachol and pilocarpine confirmed that the functional changes observed following xanomeline pretreatments were due to unique ligand-specific characteristics rather than being a general property of agonist binding to the receptor. When cells were pretreated with pilocarpine for 1 h followed by washing and waiting 23 h or for 24 h prior to washing, no changes in carbachol-induced receptor stimulation were observed. Similarly, pretreatment with carbachol for 1 h followed by washing and waiting 23 h did not alter the ability of carbachol to elicit a response. However, when cells were treated for 24 h with carbachol prior to washing, there was a depression in the maximal response in addition to a reduction in potency of the carbachol-mediated response (fig. 3). Literature supports that the observed decrease in the functional response may be due to internalization of cell-surface receptors in addition to uncoupling of the receptor from G-proteins [16, 18]. Pretreatment of cells with pilocarpine produced changes in its ability to subsequently activate the receptor similar to those described above for carbachol. Contradictory reports exist regarding the effects of prolonged exposure to pilocarpine on receptor expression [33, 34]. However, in our paradigm 24-hour exposure of cells to pilocarpine prior to washing resulted in a decrease in receptor expression (data not shown). Thus, the change in the functional response to pilocarpine can be attributed to a decrease in cell-surface receptor density following continuous agonist exposure, because a partial agonist needs near or full receptor occupancy to stimulate a maximal response [35].

Previous research has documented that prolonged exposure to an agonist can lead to receptor internalization, downregulation or uncoupling of the G-protein [15]. Experiments were therefore conducted to determine whether the observed long-term effects of xanomeline are due to receptor internalization or downregulation. These

possibilities were differentiated by comparing the effects of xanomeline treatment on the specific binding of [3 H]QNB and [3 H]NMS (fig. 4). QNB is a lipophilic antagonist that is able to bind to both cell-surface and internalized (but intact) receptors [26, 27], whereas NMS (a permanently-charged quaternary amine) is only able to bind to receptors located on the cell surface [28]. The absence of significant differences between the binding profiles of [3 H]QNB and [3 H]NMS as a result of 24-hour treatment with xanomeline prior to washing supports the notion that the effects observed in both displacement and saturation binding experiments are mainly due to receptor downregulation rather than internalization. Interestingly, treatment with xanomeline for 1 h followed by washing and waiting 23 h indicated an apparent higher potency of xanomeline in decreasing [3 H]QNB than [3 H]NMS binding. An opposite profile would have been expected if a major proportion of the receptors were being internalized rather than downregulated. One possible explanation of these data is that xanomeline wash-resistant binding to the M3 receptor may impart differential allosteric modulation of NMS and QNB binding. The magnitude of cooperativity between the orthosteric and an allosteric site on a receptor is dependent on the specific receptor conformations effected by binding of various ligands [36]. Therefore, the effects of a given allosteric modulator are ligand specific.

While xanomeline wash-resistant binding takes place at an allosteric site on the muscarinic receptor, the persistent functional response likely involves activation of the receptor primary binding domain by xanomeline's active head group [9, 12, 37]. While the formation of xanomeline wash-resistant binding takes place in the presence of atropine, the resulting wash-resistant receptor activation is blocked by atropine [12]. Thus, further experiments were conducted to determine the role of receptor activation in the long-term changes observed following acute exposure of the receptor to xanomeline (fig. 5). Blocking the primary binding site with atropine during brief treatment with xanomeline did not prevent long-term reductions in receptor availability induced by persistently-bound xanomeline, suggesting that activation of the receptor during the initial treatment period is not necessary in order for xanomeline to induce long-term changes. These data also indicate that access to the orthosteric site is not necessary for wash-resistant binding of xanomeline to take place. This is in agreement with previous studies at the M1 receptor showing that this interaction takes place at a secondary receptor binding site [12, 14, 38]. However, blocking the primary binding site

with atropine during the long waiting period following washing off free xanomeline blunted the long-term effects of xanomeline on [3 H]NMS binding. Together, these results indicate that receptor activation is necessary to produce the long-term consequences of xanomeline wash-resistant binding.

To further explore the importance of receptor activation in the long-term effects of xanomeline, receptor function was inhibited using siRNA targeted against the G_q and G_{11} G-proteins (fig. 6). Functional assays and Western blots confirmed that receptor function and detectable $G_{q/11}$ G-protein expression were significantly reduced. When siRNA-transfected cells were subjected to the various xanomeline treatments, no differences in receptor density on the cell-surface were observed. This is in contrast to non-transfected cells in which prolonged xanomeline pretreatments resulted in decreased cell-surface receptor density. These results further support the importance of receptor function in the development of xanomeline's long-term effects.

Functional experiments were conducted to determine the effects of suppressing receptor activation on the delayed actions of xanomeline (fig. 7). The concomitant presence of atropine with xanomeline during the initial pretreatment period, followed by washing and prolonged incubation in ligand-free medium, prevented the change in carbachol potency in stimulating inositol phosphate production observed following xanomeline treatment alone. This is in contrast to the results obtained in [3 H]NMS saturation binding experiments in which the long-term effects of wash-resistant xanomeline binding were still evident under these conditions. In comparison, the presence of atropine during the 23-hour incubation following acute xanomeline treatment resulted in a decrease in carbachol efficacy. Once again, this is in contrast to the observed antagonism by atropine of xanomeline-induced changes in [3 H]NMS saturation binding. These discrepancies may be due to unique interactions of atropine with the receptor. While commonly described as an antagonist, atropine also exhibits properties of an inverse agonist [39, 40]. For example, previous research found that continuous treatment with atropine can lead to an increase in receptor expression [23, 29]. Thus, the effects of atropine itself may be concealing the functional effects of long-term xanomeline exposure.

Previous experiments at the M1 receptor have shown that changing the structure of xanomeline, specifically shortening the carbon side chain, results in alterations in its persistent binding and receptor activation [31]. We confirmed that the propyl analog of xanomeline, which

has three fewer carbons in the side chain, exhibits low-potency wash-resistant binding at the M3 receptor in the virtual absence of concomitant receptor activation. Using a [³H]NMS saturation binding paradigm (fig. 8), pretreatment with the propyl analog for 1 h followed by washing and prolonged incubation resulted in a very small decrease in cell-surface receptor expression compared to that elicited by xanomeline, without a change in radioligand affinity. In contrast, 24-hour treatment with the propyl analog prior to washing resulted in an unexpected significant ($p < 0.05$) increase in cell-surface receptor density in conjunction with a large reduction in radioligand affinity. This is in stark contrast to the effects induced by xanomeline treatment for 24 h prior to washing, whereby a marked decrease in cell-surface receptor expression was observed. Taken together, these results suggest that wash-resistant binding in the absence of persistent receptor activation is not the major contributor to the long-term effects of xanomeline binding.

Additional [³H]NMS saturation binding experiments were conducted at 4°C, which has been shown to prevent receptor activation by agonists [15, 41]. Treatment of cells with xanomeline for 1 h at 37°C followed by washing and 23-hour incubation at 4°C did not result in changes in binding parameters compared to untreated control cells incubated at 4°C. However, when cells were treated with 10 or 100 µmol/l xanomeline for 24 h at 4°C prior to washing, there was a concentration-dependent decrease in radioligand affinity (fig. 9). Taken together, these results suggest that xanomeline avidly bound at the receptor allosteric site requires receptor activation for the induction of its long-term effects on receptor expression. Interestingly, while the effects of xanomeline on maximal cell-surface receptor density seem to be dependent on re-

ceptor activation at the orthosteric site, xanomeline's effects on radioligand affinity are not. This differential effect on $B_{\max}$ and K_d is consistent with other protocols that inhibit receptor function. It has to be noted, however, that changing temperature is known to elicit general changes in the conformation of many cellular proteins in addition to altering receptor conformation.

Even though xanomeline is a partial agonist at the M3 receptor [13], it exerts long-term changes in receptor regulation similar to those observed at the M1 receptor [13, 14], albeit to a lesser extent. This may suggest a shared mechanism of agonist-induced regulation for the M3 and M1 receptors. This is not surprising since functional responses at both the M3 and M1 receptors are elicited via the same class of G-proteins [1].

In summary, we have demonstrated that the long-term consequences of xanomeline binding at the M3 receptor are due to unique properties of the mode of interaction of xanomeline with the receptor. These effects of xanomeline are most likely the result of receptor downregulation, allosteric modulation of the receptor or a combination of both. Additionally, long-term receptor modulation by xanomeline is dependent on an intact functional receptor response.

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