

## Long-term wash-resistant effects of brief interaction of xanomeline at the M<sub>1</sub> muscarinic receptor

Kayla C. De Lorme, Krista L. Sikorski, Marianne K.O. Grant, Esam E. El-Fakahany\*

*Division of Neuroscience Research in Psychiatry, University of Minnesota Medical School,  
Mayo Mail Code 392, 420 Delaware St. SE, Minneapolis, MN 55455, USA*

Received 15 August 2006; received in revised form 21 September 2006; accepted 23 September 2006

### Abstract

Compared to other M<sub>1</sub> muscarinic acetylcholine receptor (M<sub>1</sub> mAChR) agonists, xanomeline demonstrates both reversible and persistent modes of binding to the receptor. In our study, we investigated the long-term consequences of brief incubation of Chinese hamster ovary cells expressing M<sub>1</sub> mAChR (M<sub>1</sub>-CHO) with low concentrations of xanomeline followed by washing off the free drug. Thus, M<sub>1</sub>-CHO cells were exposed to 100 nM xanomeline for 1 h then washed extensively. Washed cells were either used immediately for binding assays or incubated for 23 h in the absence of free xanomeline. Only the latter treatment conditions resulted in marked attenuation of binding of the muscarinic radioligand [<sup>3</sup>H]N-methylscopolamine ([<sup>3</sup>H]NMS) to intact cells. Shortening the xanomeline pretreatment period to 1 min had the same trends as the 1 h pretreatment, implying that xanomeline binds instantly to the receptor to elicit long-term wash-resistant effects. Presence of atropine during the brief period of xanomeline pretreatment did not markedly modulate xanomeline's long-term effects, which suggests that persistent anchoring of the xanomeline molecule to the M<sub>1</sub> receptor takes place at a site distinct from the orthosteric binding domain. Our findings suggest the possibility of a time-dependent transition of the conformation of the muscarinic M<sub>1</sub> receptor-xanomeline complex between states that vary in their ability to bind [<sup>3</sup>H]NMS. However, possible involvement of other mechanisms of long-term receptor regulation cannot be discounted.

© 2006 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Xanomeline; Wash-resistant binding; Muscarinic receptors; Allosteric modulation

The muscarinic acetylcholine receptor family consists of five receptor subtypes (M<sub>1</sub>–M<sub>5</sub>) and is part of the superfamily of G-protein coupled receptors [6]. Targeting the M<sub>1</sub> muscarinic acetylcholine receptor (M<sub>1</sub> mAChR) has become of interest due to its role in learning and memory, and the potential it may have in treating certain neurodegenerative diseases [10]. The search for a selective M<sub>1</sub> agonist is necessary to prevent activation of other subtypes such as the M<sub>3</sub>, which is responsible for many of the gastrointestinal side effects seen in acetylcholine replacement therapies. Because the orthosteric binding domain on muscarinic receptors where acetylcholine and conventional agonists interact is highly conserved among the five muscarinic receptor subtypes, developing a highly selective M<sub>1</sub> mAChR agonist has been difficult. However, multiple allosteric binding sites have been identified on the M<sub>1</sub> mAChR [4]. Being distant from the primary binding site, they are likely to vary in sequence

among receptor subtypes [1,4] and may serve as targets for binding of M<sub>1</sub> mAChR-selective agonists [12].

Xanomeline is a potent, functionally selective M<sub>1</sub> mAChR agonist [11]. Previous studies have demonstrated that it displays a unique mode of binding to the M<sub>1</sub> mAChR as compared to other agonists. In addition to its reversible interaction with the classical orthosteric binding site of the M<sub>1</sub> mAChR, xanomeline also binds persistently to a secondary site(s) on the receptor [2,3]. This wash-resistant binding occurs almost instantaneously at M<sub>1</sub>, but not at M<sub>2</sub> mAChR [7], which suggests induction of receptor subtype-specific conformations by xanomeline. This receptor subtype selectivity and the lack of similar effects when liposomes are treated with xanomeline prior to reconstitution of M<sub>1</sub> receptors [8] support a receptor specific mechanism. These findings also strongly argue against the possibility of membrane lipids serving as a non-specific depot of xanomeline where xanomeline leeches out to rebind to the receptor causing persistent activation. It has been shown that persistent xanomeline binding to the M<sub>1</sub> mAChR acutely modulates binding of ligands to the orthosteric site in an allosteric manner [2,7,9]. It

\* Corresponding author. Tel.: +1 612 624 8432; fax: +1 612 624 8935.  
E-mail address: [elfak001@umn.edu](mailto:elfak001@umn.edu) (E.E. El-Fakahany).

also causes wash-resistant receptor activation that is silenced by atropine [2]. However, long-term consequences of xanomeline's persistent interaction with the  $M_1$  mAChR are not known.

In the current study, we examined the long-term effects of brief exposure of the  $M_1$  mAChR to low concentrations of xanomeline. Chinese hamster ovary cells, stably expressing the human  $M_1$  mAChR ( $M_1$ -CHO), were grown at 37 °C in Dulbecco's modified Eagle's medium, supplemented with 10% bovine calf serum and 50  $\mu$ g/ml geneticin, in a humidified atmosphere consisting of 5%  $CO_2$  and 95% air.  $M_1$ -CHO cells were pretreated in monolayer in the absence or in the presence of 100 nM xanomeline at 37 °C. After short incubation times (1 h or 1 min), cells were washed three times to remove unbound xanomeline with iso-osmotic HEPES buffer (110 mM NaCl, 5.3 mM KCl, 1.8 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 25 mM glucose, 20 mM HEPES, and 58 mM sucrose, pH 7.4; 340 mOsm). Cells were then used immediately in binding assays or allowed to incubate in monolayer in the absence of free xanomeline for a long period of time (23 or 24 h at 37 °C, in the case of 1 h or 1 min pretreatment with xanomeline, respectively). In all cases, cells were washed again three times prior to their use in binding experiments. Further experiments were designed using atropine (10  $\mu$ M), either during the initial pretreatment period with xanomeline for 1 h, or during the 23 h incubation following washing off free xanomeline. Each experimental group also had respective controls in the absence of xanomeline pretreatment. Binding of [ $^3$ H]N-methylscopolamine ([ $^3$ H]NMS) was determined in intact cells (1 h, 37 °C) and radioactivity was measured using liquid scintillation counting. Non-specific binding was determined using 10  $\mu$ M atropine. Results are expressed as mean  $\pm$  standard error of the mean. Statistical significance was determined by paired or unpaired *t*-tests, as appropriate. A probability (*p*) value <0.05 was taken to indicate statistical significance.

The binding of 0.2 nM [ $^3$ H]NMS in intact  $M_1$ -CHO cells following the 1 h xanomeline pretreatment conditions is shown in Fig. 1. The continuous presence of 100 nM xanomeline in naïve cells inhibited the binding of [ $^3$ H]NMS by 34% in comparison to control untreated cells (data not shown). This experimental group detects the combined effects of both reversible and wash-resistant xanomeline binding. Pretreatment of cells with the same concentration of xanomeline for 1 h followed by washing did not demonstrate significant apparent wash-resistant effects on [ $^3$ H]NMS binding (Fig. 1, open bar). However, a marked decrease (44%) in radioligand binding compared to vehicle-treated cells was observed when pretreated cells were allowed to incubate for an additional 23 h in the absence of free xanomeline (Fig. 1, dark bar). This effect was similar to, albeit smaller than, the effect of continuous incubation of cells with 100 nM xanomeline for 24 h before washout (Fig. 1, striped bar).

In order to further examine the effects of the time dependence of xanomeline, the pretreatment time was drastically reduced from 1 h to 1 min. Surprisingly, very similar effects were seen following 1 min as compared to 1 h pretreatment conditions. Thus, while [ $^3$ H]NMS binding was not reduced by 1 min pretreatment with 100 nM xanomeline followed by washing and immediate use (Fig. 1, open bar) as compared to binding in

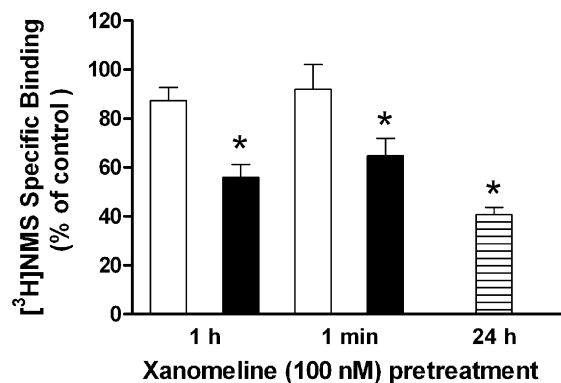


Fig. 1. Effects of xanomeline pretreatment, followed by washout on [ $^3$ H]NMS binding in  $M_1$ -CHO cells. Cells were pretreated with 100 nM xanomeline for 1 h or 1 min at 37 °C, washed extensively then used immediately in binding assays (open bars) or allowed to incubate in the absence of free xanomeline for 23 h or 24 h (dark bars), respectively. Another group of cells were continuously incubated with 100 nM xanomeline for 24 h followed by washing and immediate determination of [ $^3$ H]NMS binding (striped bar). Receptor binding was assayed by incubation of intact cells for 1 h at 37 °C with 0.2 nM [ $^3$ H]NMS. For each experimental group, control binding was defined as specific [ $^3$ H]NMS binding in vehicle-treated cells. When continuously present during the binding assay, 100 nM xanomeline resulted in 34% reduction in radioligand binding in naïve cells (data not shown). Values represent the mean  $\pm$  S.E.M. of 6–15 experiments performed in triplicate. (\*) Paired *t*-test showed a significant difference (*p* < 0.05) compared to control.

control untreated cells, a 35% reduction in radioligand binding was observed when pretreated cells were washed and allowed to incubate for an additional 24 h in the absence of free xanomeline (Fig. 1, dark bar).

Additional experiments utilizing the muscarinic antagonist atropine were designed to investigate the mechanisms underlying the long-term effects of prebound xanomeline. Namely, whether xanomeline's initial interaction with the receptor involves sites other than the orthosteric binding site, and whether receptor activation is required for subsequent long-term effects of xanomeline. We have shown previously that blockade of the receptor's primary binding domain by atropine still allows wash-resistant interaction of xanomeline with the  $M_1$  receptor [9]. However, atropine completely blocks receptor activation by prebound xanomeline [2]. Taken together, xanomeline tight binding takes place at a secondary allosteric site on the  $M_1$  muscarinic receptor, but the associated persistent receptor activation requires interaction of xanomeline's active head group with the receptor's primary binding domain. To achieve these goals, atropine was either added at a receptor-saturating concentration (10  $\mu$ M) simultaneously with xanomeline during the 1 h pretreatment period, or during the long waiting period in the absence of free xanomeline. The concentration of xanomeline used for pretreatment was increased to 3  $\mu$ M in these experiments to maximize xanomeline long-term effects and therefore facilitate observing the effects of atropine. Furthermore, a saturating concentration (6 nM) of [ $^3$ H]NMS was chosen to determine changes in maximal binding without interference from alterations in receptor affinity.

As shown in Fig. 2 (open bar), pretreatment of cells with 3  $\mu$ M xanomeline for 1 h followed by washing and waiting for

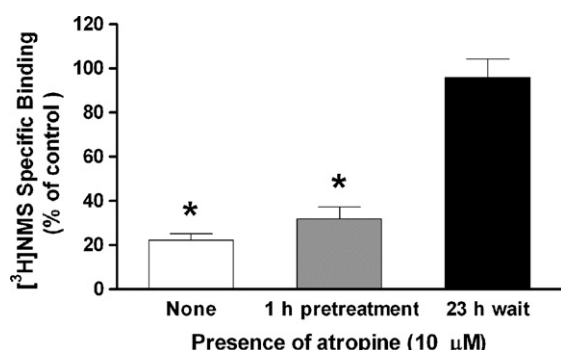


Fig. 2. Effects of atropine on the long-term effects of persistently bound xanomeline. M<sub>1</sub>-CHO cells were pretreated for 1 h at 37 °C with 3  $\mu$ M xanomeline in the absence (open bar) or in the presence (grey bar) of 10  $\mu$ M atropine, washed extensively and allowed to incubate for 23 h before determination of [ $^3$ H]NMS binding in intact cells (6 nM, 1 h at 37 °C). Another group of cells (dark bar) was preincubated with 3  $\mu$ M xanomeline for 1 h at 37 °C, washed, and then incubated with 10  $\mu$ M atropine for 23 h. Data were normalized to percent of control binding in the absence of xanomeline, but in the presence of corresponding incubations with atropine for either 1 or 23 h. Bars represent the mean  $\pm$  S.E.M. of four experiments performed in triplicate. (\*) Paired *t*-test showed a significant difference ( $p < 0.05$ ) compared to each group's respective controls in the absence of xanomeline.

23 h inhibited [ $^3$ H]NMS binding by 78% (compared to vehicle-treated cells). When cells were pretreated with xanomeline and atropine simultaneously for 1 h, followed by washing and waiting for 23 h in the absence of either drug, the inhibition of [ $^3$ H]NMS binding was only slightly reduced to approximately 68% of control binding (Fig. 2, grey bar). Thus, 10% of radioligand binding was recovered when atropine was present during the pretreatment. More evident was the effect of atropine when present only during the 23 h incubation period after xanomeline pretreatment and washing. These conditions completely obliterated the long-term effects of xanomeline prebound to the receptor (Fig. 2, dark bar). Control binding was defined as pretreatment in the absence of xanomeline, but in the presence of atropine for the corresponding incubation periods of either 1 or 23 h.

Complete blockade of the orthosteric site by atropine during pretreatment with xanomeline only slightly suppressed the long-term effects of xanomeline. These results are in concert with our previous demonstration of the possible simultaneous interaction of xanomeline (at the allosteric site) and atropine (at the orthosteric site) with the M<sub>1</sub> mAChR [9]. When atropine is washed away, persistently bound xanomeline continues to elicit long-term changes of the receptor. In contrast, the presence of atropine during the long-term incubation period completely abolished the effects of wash-resistant xanomeline on radioligand binding (Fig. 2), suggesting an interaction involving xanomeline's active head group with the receptor's primary binding domain as suggested by our previous findings [2].

One plausible interpretation of our data is that brief incubation with low concentrations of xanomeline results in initial wash-resistant binding at a distinct receptor domain on the muscarinic M<sub>1</sub> receptor to induce a receptor conformation where the orthosteric binding domain is still fully capable of recognizing [ $^3$ H]NMS. This initial conformation is then transformed

in a time-dependent manner to one that exhibits reduced binding of [ $^3$ H]NMS. It is worth noting that very similar effects were seen following 1 min as compared to 1 h pretreatment conditions (Fig. 1), suggesting that induction of the initial xanomeline-receptor conformation is immediate. Receptor internalization or down-regulation may also explain the delayed large decrease in binding effected by persistently bound xanomeline. There is a large body of evidence suggesting that long-term treatment with agonists can decrease receptor expression or diminish receptor-mediated functional responses [5,13,14]. This notion is supported by the similarity in the effects of prolonged incubation of xanomeline pretreated and washed cells and those of continuous incubation with xanomeline for 24 h (Fig. 1). Detailed future experiments utilizing cell-permeable muscarinic radioligands, Western analysis of receptor expression and broken cell preparations are required to differentiate among these mechanistic possibilities.

In this study we have clearly shown that very brief exposure of M<sub>1</sub>-CHO cells to low concentrations of xanomeline that do not cause immediate wash-resistant effects on ligand binding results in long-term effects on the M<sub>1</sub> mAChR. These effects are time-dependent and involve binding of xanomeline at a secondary allosteric domain on the receptor. Future research is planned to determine the molecular mechanisms involved in this phenomenon.

## Acknowledgement

This work was supported by NIH grant NS25743.

## References

- [1] T.I. Bonner, W.S. Modi, H.N. Seunanez, S.J. O'Brien, Chromosomal mapping of the five human genes encoding muscarinic acetylcholine receptors, *Cytogenet. Cell Genet.* 58 (1991) 1850–1851.
- [2] A. Christopoulos, T.L. Pierce, J.L. Sorman, E.E. El-Fakahany, On the unique binding and activation properties of xanomeline at M<sub>1</sub> muscarinic acetylcholine receptor, *Mol. Pharmacol.* 53 (1998) 1120–1130.
- [3] A. Christopoulos, E.E. El-Fakahany, Novel persistent activation of muscarinic M<sub>1</sub> receptors by xanomeline, *Eur. J. Pharmacol.* 334 (1997) R3–R4.
- [4] L.M. Espinoza-Fonseca, J.G. Trujillo-Ferrara, The existence of a second allosteric site on the M<sub>1</sub> muscarinic acetylcholine receptor and its implications for drug design, *Bioorg. Med. Chem. Lett.* 16 (2006) 1217–1220.
- [5] J. Hu, S.Z. Wang, E.E. El-Fakahany, Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in Chinese hamster ovary cells, *J. Pharmacol. Exp. Ther.* 257 (1991) 938–945.
- [6] E.C. Hulme, N.J.M. Birdsall, N.J. Buckley, Muscarinic receptor subtypes, *Ann. Rev. Pharmacol. Toxicol.* 30 (1990) 633–673.
- [7] J. Jakubík, E.E. El-Fakahany, V. Doležal, Differences in kinetics of xanomeline binding and selectivity of activation of G proteins at the M<sub>1</sub> and M<sub>2</sub> muscarinic acetylcholine receptor, *Mol. Pharmacol.* 70 (2006) 656–666.
- [8] J. Jakubík, S. Tuček, E.E. El-Fakahany, Role of receptor protein and membrane lipids in xanomeline wash-resistant binding to the muscarinic M<sub>1</sub> receptors, *J. Pharmacol. Exp. Ther.* 308 (2004) 105–110.
- [9] J. Jakubík, S. Tuček, E.E. El-Fakahany, Allosteric modulation by persistent binding of xanomeline of the interaction of competitive ligands with the M<sub>1</sub> muscarinic acetylcholine receptor, *J. Pharmacol. Exp. Ther.* 301 (2002) 1033–1041.
- [10] M. McKinney, J.T. Coyle, The potential for muscarinic receptor subtype-specific pharmacotherapy for Alzheimer's disease, *Mayo Clin. Proc.* 66 (1991) 225–237.

- [11] H.E. Shannon, F.P. Bymaster, D.O. Calligaro, B. Greenwood, C.H. Mitch, B.D. Sawyer, J.S. Ward, D.T. Wong, P.H. Olesen, M.J. Sheardown, M.D.B. Swedberg, P.D. Suzdak, P. Sauerberg, Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M<sub>1</sub> receptors, *J. Pharmacol Exp. Ther.* 269 (1994) 271–281.
- [12] T.A. Spalding, C. Trotter, N. Skjaerbaerk, T.L. Messier, E.A. Currier, E.S. Burstein, D. Li, U. Hacksell, M.R. Brann, Discovery of an ectopic activation site on the M1 muscarinic receptor, *Mol. Pharmacol.* 61 (2002) 1297–1302.
- [13] J.K. Walker, R.R. Gainetdinov, D.S. Feldman, P.K. McFawn, M.G. Caron, R.J. Lefkowitz, R.T. Premont, J.T. Fisher, G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation, *Am. J. Physiol. Lung Cell Mol. Physiol.* 286 (2004) L312–L319.
- [14] S.Z. Wang, J.R. Hu, R.M. Long, W.S. Pou, C. Forray, E.E. El-Fakahany, Agonist-induced down-regulation of m1 muscarinic receptors and reduction of their mRNA level in a transfected cell line, *FEBS Lett.* 276 (1990) 185–188.