The action of xanomeline on human muscarinic receptors expressed in Chinese hamster ovary cells

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

MEREDITH NOETZEL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Dr. Esam El-Fakahany

JANUARY, 2009

© Meredith Noetzel, January 2009

Acknowledgements

I would like to thank my advisor Dr. Esam El-Fakahany for his support and guidance. He kindly let me join his lab and be his last graduate student. He has also helped me to grow as a scientist.

I would like to thank my committee members Dr. Virginia Seybold, Dr. William Engeland and Dr. Ping Law for their time and advice. Without their thoughtful input I would not have been able to complete my thesis as quickly. They have also been instrumental in helping me learn to present my work in a clear and concise manner.

The El-Fakahany lab members that I have worked with have been great. I would especially like to thank Marianne Grant for all of her help and support. She was always there to teach me new techniques, answer questions and help with anything. Without her I would not have been able to get everything done. I would also like to thank Kayla De Lorme and Allison Cherry for the great conversions and making the lab a fun place to be. I would also like to acknowledge both Marianne Grant and Kayla De Lorme for providing some of the data presented in this thesis.

I would also like to thank my former lab. Dr. Walter Low has been a great colleague over the years. I would specifically like to thank Terry Burns for his help and thoughtful discussions. I would also like to thank Aleta Rees, David Horn and Yuliya Perepelitsa for their hard work and helping me learn to be a better mentor.

Lastly, I would like to thank my friends and family for supporting me through all of my schooling. They have always been there when I needed them, in both the good times and the bad. I would especially like to thank my parents for their support and encouragement over the years. I would like to thank my boyfriend, Mark Gramith, who has helped me through each stage and has had an incredible amount of patience. I

would also like to thank Thaddeus Brink for taking the time to read through my thesis and giving my feedback.

This dissertation is dedicated to my grandfather David Noetzel,
who got me interested in science at a very young age.

Thesis abstract

Muscarinic acetylcholine receptors are part of the G protein-coupled superfamily of receptors. There are five subtypes of muscarinic receptors (M1-M5). These receptors are therapeutic targets for in a number of diseases, including Alzheimer's disease and schizophrenia. The orthosteric (primary) binding domain of muscarinic receptors is highly conserved across subtypes making it difficult to develop agonists or antagonists that bind with selectivity at a particular subtype. However, there are allosteric (secondary) binding sites on muscarinic receptors that are thought to be less well conserved between subtypes, which may lead to the development of drugs that bind in a subtype-selective manner. One potential drug of interest is xanomeline. Previous research has found that it binds in a unique manner to muscarinic receptors; it binds in a reversible manner at the orthosteric site and in a wash-resistant manner at an allosteric site. Furthermore, xanomeline is thought to be an M1/M4 selective agonist. The goal of the current research was to characterize the effects of prolonged exposure of the various receptor subtypes to xanomeline and determine the mechanisms through which these effects occur. To do this Chinese hamster ovary cells expressing the various individual muscarinic receptors were exposed to xanomeline for brief and long-term time periods and radioligand binding and functional assays were performed. Xanomeline was shown to bind in a reversible and wash-resistant manner at the M1-M4 receptor subtypes. Although the effects of acute xanomeline exposure were similar across subtypes, long-term treatment with xanomeline resulted in differential effects among subtypes. More detailed experiments were conducted at the M1 and M3 receptor subtypes to elucidate the mechanisms of the long-term effects of xanomeline. Wash-resistant xanomeline binding was able to modulate the functional properties of the receptor. The consequences of the long-term effects of wash-resistant xanomeline binding are

dependent on activation of the receptor through the orthosteric binding site. It appears that these effects are a result of receptor internalization and allosteric modulation of the receptor. Thus, my research demonstrates that it is important to determine both the acute and long-term drug interactions with the receptor as part of the drug development process.

TABLE OF CONTENTS

Chapter 1: Introduction – muscarinic receptors and xanomeline

1. Overview 2
2. G protein-coupled receptors 4
3. Subtypes, localization and function of muscarinic receptors 5
4. Structure and binding of muscarinic receptors 7
5. A model of receptor activation 9
6. Receptor trafficking 10
7. Drug-receptor interactions 10
8. Signaling pathways for muscarinic receptors 11
9. Consequences of prolonged exposure of muscarinic receptors to agonist and antagonists 15
10. Influence of receptor mutations and expression levels on receptor activity 20
11. In vitro receptor expression systems 21
12. Allosteric interactions with the receptor 23
13. Pharmacological profile of allosteric binding 24
14. Allosteric sites on muscarinic receptors 25
15. Muscarinic receptors, allosteric sites and diseases 28
16. Alzheimer's disease 28
17. Cholinergic hypothesis of Alzheimer's disease 29
18. Cholinergic treatments for Alzheimer's disease 31
19. Non-cholinergic treatments for Alzheimer's disease 32
20. Schizophrenia 33
21. Treatment for schizophrenia 35
22. Binding and functional properties of xanomeline 35
23. Proposed mechanism of xanomeline binding 37
24. Wash-resistant xanomeline binding 38
25. Xanomeline as a treatment for Alzheimer's disease and schizophrenia 39
26. Xanomeline treatment in Alzheimer's disease models 40
27. Clinical trials with xanomeline for the treatment of Alzheimer's disease 41
28. Xanomeline treatments in animal models of schizophrenia 42
29. Clinical trials with xanomeline for the treatment of Alzheimer's disease 43
30. Summary 43

Chapter 2: Materials and methods

1. Materials and general treatment protocols 46
2. Radioligand binding assays 48
3. Functional assays 50
4. Variations on radioligand binding and functional assay paradigms 54
5. Data analysis 59

Chapter 3: Determination of the acute and long-term effects of xanomeline binding at M1-M4 muscarinic receptors

1. Introduction 61
2. Results 62
3. Discussion 72
4. Figures 80
5. Tables 115

Chapter 4: Determination of the acute and long-term functional effects of xanomeline at the M1 and M3 muscarinic receptors
1. Introduction 121
2. Results 122
3. Discussion 131
4. Figures 136
5. Tables 152

Chapter 5: Receptor activation is required for the development of the long-term effects of xanomeline at the M1 and M3 muscarinic receptors
1. Introduction 158
2. Results 159
3. Discussion 167
4. Figures 172
5. Tables 188

Chapter 6: Mechanisms of the long-term effects of xanomeline wash-resistant binding
1. Introduction 196
2. Results 197
3. Discussion 204
4. Figures 210
5. Tables 219

Chapter 7: Conclusions 224

References 230

Appendix A: Differences observed between suspension and monolayer protocols
1. Introduction 242
2. Results 242
3. Discussion 244
4. Figures 246
5. Table 248

LIST OF TABLES

Chapter 3: Determination of the acute and long-term effects of xanomeline binding at M1-M4 muscarinic receptors

Table 1. Short- and long-term effects of xanomeline, carbachol or pilocarpine binding on [^{3}H]NMS binding in CHO cells expressing hM1-hM4 receptor subtypes. 115

Table 2. [^{3}H]NMS saturation binding parameters for xanomeline, carbachol or pilocarpine pretreatment and waiting periods in CHO cells expressing hM1-hM4 receptor subtypes. 118

Chapter 4: Determination of the acute and long-term functional effects of xanomeline at the M1 and M3 muscarinic receptors

Table 1. Effects of agonist treatment on PI hydrolysis in CHO cells expressing hM1 and hM3 receptor subtypes. 152

Table 2. Effects of carbachol, pilocarpine or xanomeline stimulation following short- and long-term agonist pretreatment and washout on inositol phosphates production in CHO cells expressing hM3 receptors. 153

Table 3. Effects of carbachol, pilocarpine or xanomeline stimulation following short- and long-term agonist pretreatment and washout on inositol phosphates production in CHO cells expressing hM1 receptors. 155

Chapter 5: Receptor activation is required for the development of the long-term effects of xanomeline at the M1 and M3 muscarinic receptors

Table 1. Effects of xanomeline pretreatment and washout in the absence or presence of atropine on [^{3}H]NMS saturation binding and functional parameters of carbachol stimulation in CHO cells expressing hM1 or hM3 receptors. 188

Table 2. Effects of xanomeline pretreatment on inhibition of NMS binding, saturation binding and functional parameters in wild-type and mutant rM1 receptors expressed in CHO cells. 190

Table 3. Effects of blocking receptor function using siRNA against G_q and G_{11} G 192 proteins on [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors assayed in suspension.

Table 4. Effects of xanomeline analogs on inhibition of [^{3}H]NMS binding and functional parameters in CHO cells expressing hM3 receptors. 193

Table 5. Effects of treatment with the propyl xanomeline analog on [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors. 194

Chapter 6: Mechanisms of the long-term effects of xanomeline wash-resistant binding

Table 1. Inhibition of radioligand binding using a receptor-saturating concentration of [^{3}H]QNB or [^{3}H]NMS following xanomeline pretreatment and various waiting periods in CHO cells expressing hM1 or hM3 receptor subtypes. 219

Table 2. Effects of blocking receptor activation on xanomeline-induced changes in [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors. 221

Table 3. Effects of changing the level of receptor expression or inactivating the G proteins on xanomeline-induced changes in [^{3}H]NMS saturation binding parameters in CHO cells expressing the hM4 receptor subtype. 222

Appendix A: Differences observed between suspension and monolayer protocols

Table 1. Effects on [^{3}H]NMS saturation binding parameters when CHO hM3 cells were assayed in monolayer versus suspension. 248

LIST OF FIGURES

Chapter 3: Determination of the acute and long-term effects of xanomeline binding at M1-M4 muscarinic receptors

Figure 1. Time course of the development of xanomeline wash-resistant binding at hM1-hM4 muscarinic receptor subtypes expressed in CHO cells. 80

Figure 2. Inhibition of [^{3}H]NMS binding for acute xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 82

Figure 3. Effects of acute DMSO pretreatments on the inhibition of [^{3}H]NMS binding in CHO cells stably expressing hM1-hM4 receptor subtypes. 84

Figure 4. Effects of acute xanomeline pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 86

Figure 5. Effects of acute DMSO pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 88

Figure 6. Inhibition of [^{3}H]NMS binding for acute carbachol pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 90

Figure 7. Effects of acute carbachol pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 92

Figure 8. Inhibition of [^{3}H]NMS binding for acute pilocarpine pretreatments in CHO cells stably expressing the hM3 receptor subtype. 94

Figure 9. Effects of acute pilocarpine pretreatments on protein content in CHO cells stably expressing the hM3 receptor subtype. 95

Figure 10. [^{3}H]NMS saturation binding subsequent to acute xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 96

Figure 11. Inhibition of [^{3}H]NMS binding for long-term xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 98

Figure 12. Effects of long-term DMSO pretreatments on the inhibition of [^{3}H]NMS binding in CHO cells stably expressing hM1-hM4 receptor subtypes. 100

Figure 13. Effects of long-term xanomeline pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 102

Figure 14. Effects of long-term DMSO pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 104

Figure 15. Inhibition of [^{3}H]NMS binding for long-term carbachol pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 106

Figure 16. Effects of long-term carbachol pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. 108

Figure 17. Inhibition of [^{3}H]NMS binding for long-term pilocarpine pretreatments in CHO cells stably expressing the hM3 receptor subtype. 110

Figure 18. Effects of long-term pilocarpine pretreatments on protein content in CHO cells stably expressing hM3 receptor subtype. 111

Figure 19. [^{3}H]NMS saturation binding subsequent to long-term xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. 112

Figure 20. [^{3}H]NMS saturation binding subsequent to long-term agonist pretreatments in CHO cells stably expressing the hM3 receptor subtype. 114

Chapter 4: Determination of the acute and long-term functional effects of xanomeline at the M1 and M3 muscarinic receptors

Figure 1. hM3 receptor activation by xanomeline reversible and wash-resistant binding. 136

Figure 2. hM1 receptor activation by xanomeline reversible and wash-resistant binding. 137

Figure 3. Effects of acute xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM3 receptor. 138

Figure 4. Effects of acute xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM1 receptor. 140

Figure 5. Effects of acute pilocarpine on the ability of pilocarpine to subsequently stimulate the production of inositol phosphates at the hM3 receptor. 142

Figure 6. Stimulation of inositol phosphate production by exogenous phospholipase C following brief xanomeline pretreatments in cells expressing hM1 and hM3 receptors. 143

Figure 7. Stimulation of inositol phosphate production by exogenous phospholipase C following long-term xanomeline pretreatments in cells expressing hM1 and hM3 receptors. 144

Figure 8. Effects of prolonged xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM3 receptor. 145

Figure 9. Effects of prolonged xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM1 receptor. 147

Figure 10. Effects of long-term agonist exposure on the ability of various muscarinic agonists to stimulate inositol phosphates production at the hM1 and hM3 receptors. 149

Figure 11. Effects of long-term exposure to carbachol on the ability of xanomeline to stimulate inositol phosphate production at hM1 and hM3 receptors. 151

Chapter 5: Receptor activation is required for the development of the long-term effects of xanomeline at the M1 and M3 muscarinic receptors

Figure 1. Effects of atropine on long-term xanomeline-induced inhibition of [^{3}H]NMS binding in CHO cells expressing hM3 receptors. 172

Figure 2. Effects of atropine on long-term xanomeline-induced inhibition of $[^3H]$NMS binding in CHO cells expressing hM1 receptors. 174

Figure 3. Effects of atropine on long-term modulation of inositol phosphates production by xanomeline in CHO cells expressing hM3 receptors. 176

Figure 4. Effects of atropine on long-term modulation of inositol phosphates production by xanomeline in CHO cells expressing hM1 receptors. 178

Figure 5. Effects of using a non-functional receptor mutant on function, inhibition of $[^3H]$NMS and saturation binding in CHO cells expressing rM1 receptors. 180

Figure 6. Control experiments to determine $[^3H]$NMS binding and protein content following xanomeline or DMSO pretreatments in CHO cells expressing rM1 receptors. 182

Figure 7. Effects of blocking receptor function using siRNA against G_q and G_{11} G proteins on long-term xanomeline-induced inhibition of $[^3H]$NMS binding in CHO cells expressing the hM3 receptor. 184

Figure 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 hM3 receptors. 186

Chapter 6: Mechanisms of the long-term effects of xanomeline wash-resistant binding

Figure 1. Comparison of the effects of xanomeline treatment on the specific binding of $[^3H]$QNB and $[^3H]$NMS in CHO cells expressing hM3 receptors. 210

Figure 2. Comparison of the effects of xanomeline treatment on the specific binding of $[^3H]$QNB and $[^3H]$NMS in CHO cells expressing hM1 receptors. 212

Figure 3. 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 hM3 receptors. 214

Figure 4. Time course of switching of the pharmacological profile of xanomeline from an agonist to an antagonist in CHO cells expressing hM1 or hM3 receptors. 215

Figure 5. Effects of decreasing receptor expression using PrBCM on saturation binding parameters in CHO cells expressing hM4 receptors. 217

Figure 6. Effects of blocking the $G_{i/o}$ G proteins on the ability of xanomeline to induce long-term changes in CHO cells expressing the hM4 receptor. 218

Appendix A: Differences observed between suspension and monolayer protocols

Figure 1. Comparison between [^{3}H]NMS saturation binding paradigms on assays conducted in suspension versus in monolayer in CHO cells stably expressing hM3 receptors. 246

Figure 2. Comparison between inhibition of [^{3}H]NMS binding following xanomeline pretreatments for assays conducted in suspension versus in monolayer in CHO cells stably expressing hM1 receptors. 247

CHAPTER 1: Introduction

Overview

Muscarinic acetylcholine receptors are known to be involved in a variety of diseases such as Alzheimer's disease, schizophrenia, epilepsy, Parkinson's disease and depression. However, treatment of these diseases with cholinergic agents has had limited success due to side effects as a result of their non-selective binding to multiple subtypes of muscarinic acetylcholine receptors. One of the most difficult hurdles in developing agonists or antagonists that bind to muscarinic receptors with subtype selectivity is due to the highly conserved nature of the orthosteric (primary) binding domain. New research is beginning to focus on allosteric (secondary) binding sites as drug targets because these sites are less conserved across subtypes, suggesting they may lead to discovery or designing of drugs with greater subtype selectivity. Continued research is needed to learn more about these allosteric sites and the interaction of potential drugs with these sites.

Xanomeline is a novel muscarinic receptor agonist that has been shown to bind at both the orthosteric site and at an allosteric site. The major goal of this research is to characterize the actions of prolonged exposure of xanomeline at the various subtypes of muscarinic receptors. It is important to compare the long-term actions of xanomeline in the same type of cells that express individual subtypes of muscarinic receptors. This avoids complications caused by factors not related to the type of muscarinic receptor expressed, e.g. type and level of G proteins. It is also important to compare the ability of long-term treatment with xanomeline to desensitize and down-regulate various subtypes of muscarinic receptors. Differential rates or extents of receptor desensitization could contribute to agonist selectivity under chronic treatment conditions. Thus, experiments have been designed to test the hypotheses that a) xanomeline binds to all receptor subtypes in a reversible and wash-resistant manner; b) there is subtype selectivity in the

long-term effects of wash-resistant xanomeline binding; and c) xanomeline wash-resistant binding results in long-term effects similar to those seen during prolonged incubation with conventional agonists, such as internalization and down-regulation.

This introduction will address various topics that are relevant to muscarinic receptor biology and the novel muscarinic receptor agonist xanomeline, including structure, localization and function of the muscarinic receptor; the consequences of prolonged exposure to agonists; the concept of allosteric modulation of receptors; and the past and present potential for xanomeline as a treatment for Alzheimer's disease and schizophrenia.

This thesis will address the proposed hypotheses by examining the acute and long-term effects of xanomeline binding across M1-M4 receptor subtypes, the acute and long-term effects of xanomeline on function at the M1 and M3 receptor subtypes, the role of the orthosteric binding site in the long-term effects of xanomeline at the M1 and M3 receptor subtypes, and basic information about the mechanism of the long-term effects of xanomeline.

The results will provide evidence that xanomeline binds in a reversible and wash-resistant manner at all of the muscarinic receptor subtypes tested. However, there are some distinct differences in the long-term effects of xanomeline binding. Additionally, xanomeline binding is able to stimulate a functional response both in a reversible and wash-resistant manner. Interestingly, the long-term effects of exposure to wash-resistant xanomeline binding at an allosteric site are dependent on subsequent activation of the receptor at the orthosteric site. The long-term changes that are observed appear to be a result of receptor down-regulation. Further elucidation of the mode of interaction of xanomeline with various muscarinic receptor subtypes and the underlying mechanisms may lead to a better understanding of muscarinic receptor

biology. This in turn may lead to the eventual development of both muscarinic agonists and antagonists with long duration of action and perhaps better pharmacological selectivity.

G protein-coupled receptors

Muscarinic acetylcholine receptors (mAChR) are part of the large G protein-coupled receptor superfamily. The G protein-coupled receptors are one of the largest families of receptors (Wess, 2005) and contain hundreds of distinct receptors (Lefkowitz et al., 1993). Endogenous ligands for the various G proteins-coupled receptors include molecules such as biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and proteases (Gether, 2000). There are three subfamilies of G protein-coupled receptors including family A or receptors related to rhodopsin and β_2 adrenergic receptors; family B or receptors related to glucagons; and family C or receptor related to metabotropic neurotransmitter receptors (Gether, 2000). Family A is the largest of the families and the family to which muscarinic receptors belong (Gether, 2000). The molecular models of family A G protein-coupled receptors are based on rhodopsin which was the first structure crystallized (Hulme et al., 2003).

There appears to be a conservation of properties between each of the major receptor subfamilies that are coupled to G proteins that include characteristics such as amino acid sequences, topographical organization in the plasma membrane, coupling of the receptor to effectors and regulation of kinases (Lefkowitz & Caron, 1988). Interestingly, when the domains of the receptor are expressed individually they have the ability to fold on their own and then become associated to form a functional receptor (Wess, 1996).

Subtypes, localization and function of muscarinic receptors

There are five subtypes of muscarinic receptors denoted M1-M5 (Caulfield & Birdsall, 1998). Muscarinic receptors are expressed both in the brain and the periphery. In the periphery, muscarinic receptors have been shown to play a role in smooth muscle contraction, glandular secretion, and cardiac rate and force (Caulfield & Birdsall, 1998). Muscarinic receptors in the central nervous system have a wide variety of functions including playing a role in sensory, cognitive, motor, behavior, and autonomic functions (Caulfield & Birdsall, 1998; Eglen, 2005; Wess, 2004). The localization of different muscarinic receptors in the brain has been determined and knockout mice have been constructed to study the function of the individual receptor subtypes. The M1, M3 and M5 receptors are primarily expressed postsynaptically, whereas the M2 and M4 receptors are generally expressed on the presynaptic neuron (Volpicelli & Levey, 2004).

The M1 receptor is expressed in the forebrain, including the cerebral cortex, hippocampus and striatum (Wess et al., 2003). The M1 receptor is involved in cognitive processing and memory (Eglen, 2005). M1 knockout mice have an increase in locomotor behavior and some deficits in working memory (Wess et al., 2003). Based on research by Anagonstaras el al (2003), it seems that M1 receptors are not required for memory formation, but are involved in processes that require interactions between the hippocampus and cerebral cortex. This suggests that M1 receptors appear to be involved in working memory and remote reference memory (Anagnostaras et al., 2003).

The M2 receptor is expressed widely in the central nervous system and in the heart and smooth muscle (Wess, 2004; Wess et al., 2003). Autoinhibition of acetylcholine release is mediated by M2 receptors in the hippocampus and cerebral cortex (Wess et al., 2003). The M2 receptor is known to play a role in agonist-induced analgesia (Wess et al., 2003). Mice that have the M2 receptor knocked out show a

decrease in smooth muscle contraction and a decrease in body temperature (Wess et al., 2003).

M3 receptors are expressed widely throughout the brain, but at lower levels than other subtypes (Eglen, 2005; Wess et al., 2003). They are expressed at high levels in the mouse hypothalamus (Wess et al., 2003). M3 receptors are also expressed in salivary glands and peripheral organs that are innervated by parasympathetic nerves (Wess, 2004; Wess et al., 2003). The M3 receptors are known to be involved in salivation, food intake, and the gastrointestinal tract (Eglen, 2005). They have also been shown to be involved in pupilary sphincter muscle contractility (Wess, 2003). Mice in which the M3 receptor has been knocked out demonstrate decreases in body weight, food intake, serum leptin levels and serum insulin levels (Yamada et al., 2001), but an increase in energy expenditure (Gautam et al., 2006). Interestingly, the knock out mice also do not have gastrointestinal problems with absorption or motility (Gautam et al., 2006).

The M4 receptor is expressed in the forebrain (Wess, 2004), neostriatum and the ventral spinal cord (Messer, 2002). The M4 receptor is involved in autoinhibition of acetylcholine release in the striatum (Wess et al., 2003). Similar to the M2 receptor, the M4 receptor is known to be involved in agonist-induced analgesia (Wess et al., 2003). The M4 receptors have also been shown to play a role in dopamine release in the brain (van Koppen & Kaiser, 2003).

The M5 receptor is found in both neuronal and non-neuronal cells (Wess, 2004). The M5 receptor is involved in facilitating dopamine release in the striatum (Wess et al., 2003). It appears that the expression of the M5 receptors parallels, at least to some degree, dopaminergic transmission (Eglen, 2005). In addition, the M5 receptor appears to be involved in dilation of cerebral arteries and arterioles (Wess, 2004).

Structure and binding of muscarinic receptors

The structure of muscarinic receptors is similar across all five subtypes. Muscarinic receptors have seven transmembrane domains. The N-terminus is located extracellularly and contains multiple glycosylation sites. Interestingly, this region is not required for expression or function of the receptor (Caulfield & Birdsall, 1998). In contrast, the C-terminus is located intracellularly. The third cytoplasmic domain of the receptor interacts with the G protein (Caulfield & Birdsall, 1998; Volpicelli & Levey, 2004). Muscarinic receptors are thought to have over ninety percent conservation in the transmembrane domains between subtypes (Felder, 1995).There is a higher degree of similarity compared to the loops or the C- or N-terminus (Dohlman et al., 1991). There is also a high degree of homology between the orthosteric (primary) binding region across muscarinic receptor subtypes (Eglen, 2005).

A variety of molecules have been shown to bind to all five muscarinic receptors in a non-subtype selective manner. These include the endogenous ligand acetylcholine and other compounds including, but not limited to, muscarine, carbachol, pilocarpine, oxotremorine, scopolamine, and atropine. For example, the affinity of [^{3}H]N-methylscopolamine was found to be similar across muscarinic receptor subtypes expressed in CHO cells (Christopoulos et al., 1999), demonstrating binding at the orthosteric site in a non-subtype selective manner.

The residues that appear to be important in the binding of positively charged ligands, such as acetylcholine at the primary binding site, are aspartate 147, tyrosine 148, tyrosine 506, tyrosine 529 and tyrosine 533 (Han et al., 2005; Wess, 1996). Another study suggests that aspartate 113 in transmembrane domain 3 is important in the binding of acetylcholine (Gether, 2000). Additional amino acids are also thought to

play a role in ligand binding (Wess, 1996). However, it is also possible that different agonists may not share an identical binding site on the receptor (Gether, 2000).

Ligand binding is thought to result in conformational changes in the receptor. Activation of muscarinic receptors is thought to occur through an interplay between transmembrane domains 3, 5, 6 and 7 (Eglen, 2005; Wess, 1996). Research was conducted to determine the specific conformation changes of the receptor when an agonist binds and activates it. Based on a study using cross-linking of cysteine resides to determine the proximity of the external portions of the transmembrane domains to each other, it was determined that the proximity of transmembrane domains 3 and 7 increases when the receptor is activated with carbachol (Han et al., 2005). Another study using cross-linking of cysteine residues found that the cytoplasmic transmembrane domains 5 and 7 move closer together upon agonist activation (Ward et al., 2002). Similar results were seen with the β-2 adrenergic receptor, suggesting that the mechanism of receptor activation may be conserved across class A G protein-coupled receptors (Ward et al., 2002).

Thus far, orthosteric muscarinic agonists generally have been found to have a low degree of selectivity between subtypes (Wess, 2004). Some muscarinic antagonists, however, have been shown to exhibit significant selectivity among muscarinic receptor subtypes. The commonly larger size of antagonist molecules provides accessory binding to additional receptor domains that vary in their sequence from one subtype to another. For example, pirenzepine is a muscarinic antagonist that is thought to have some selectivity for the M1 receptor compared to other muscarinic receptor subtypes (Wess, 1996).

In addition to the orthosteric binding site, all subtypes are thought to have allosteric binding sites which may be less conserved between subtypes (Eglen, 2005).

The allosteric site is spatially distinct from the site where the endogenous ligand binds (Birdsall & Lazareno, 2005). The potential for a compound that acts with more subtype selectivity through an allosteric site will be discussed further below.

A model of receptor activation

The binding of an agonist to the receptor can be theoretically modeled. One type of model, the two-state model also referred to as the ternary model of binding, posits that the receptor exists in two conformations; an inactive conformation and an active conformation (Gether, 2000). In this model, the agonists are able to bind to receptors in the active conformation and thus shift the equilibrium toward that of activated receptors. Antagonists bind to both the inactive and active conformations, resulting in no change in equilibrium. In contrast, inverse agonists bind to the inactive form of the receptor resulting in a shift in equilibrium towards inactive receptors (Gether, 2000). This is quantified by the cooperativity factor α, which is based on the affinity of the ligand for the active state of the receptor compared to the affinity for the inactive state (Christopoulos & Kenakin, 2002; Hulme et al., 2003). This model is taken further in the extended ternary complex model to incorporate binding of G proteins to the receptor (Hulme et al., 2003). In this case, the receptor again exists in inactive and active conformations. However, the receptor in the active state (either with bound or no ligand) now binds to the G protein (Hulme et al., 2003). It should be noted that the G protein could also bind to the inactive form of the receptor (Christopoulos & Kenakin, 2002). In this model it is also possible that multiple G proteins have the ability to bind to the active receptor conformation, which would allow for potential specificity in receptor signaling (Kenakin, 2003).

Receptor trafficking

There may be some specificity in the way that different agonists activate the receptors resulting in activation of specific pathways even when the receptors are coupled to the same G proteins (Eglen, 2005). In the literature this has been referred to as “receptor trafficking”, “stimulus trafficking”, “functional selectivity”, “biased agonist” and “ligand induced differential signaling” (Kenakin, 2003; Urban et al., 2007). In this model, the receptor would exist in various active conformations and the binding of an agonist would have a specific effect on the conformation of the receptor, which ultimately could result in the activation of different G proteins or signaling cascades (Kenakin, 2003; Urban et al., 2007). If this occurs, it is likely that different agonists interact with the receptor in a way that breaks interactions between domains of the receptor in a unique manner upon activation (Urban et al., 2007). Support for this type of receptor-signaling interaction comes from the observation that different agonists have different rank-orders of intrinsic efficacy for the activation of multiple G proteins (Kenakin, 2003). Additionally, the presence of multiple active states of the receptor is supported by an agonist acting as a partial agonist in one setting and an inverse agonist in another setting when the same receptor is expressed (Kenakin, 2003; Urban et al., 2007). The difference in the ability of agonists to induce receptor internalization further supports this concept (Urban et al., 2007). Targeting activation of the receptor through specific conformations could be a potential strategy for drug development for the treatment of various diseases (Kenakin, 2003).

Drug-receptor interactions

Molecules bind to the receptor in a specific manner to activate (agonists), block (antagonists) or decrease (inverse agonists) receptor activation. Agonists have both

affinity and intrinsic efficacy for the receptor, whereas antagonists have only affinity (Christopoulos & El-Fakahany, 1999). An inverse agonist has negative efficacy (Christopoulos & El-Fakahany, 1999). The action of an agonist is dependent on the system that it is being studied, thus it can only be defined as a partial or full agonist for the system in which the response is measured (Christopoulos & El-Fakahany, 1999). When a system has spare receptors this means that only a fraction of the receptors needs to be activated to get a maximal response. Additionally, it is important to note that a receptor saturating concentration of a drug in one system may not be saturating in a different system (Christopoulos & El-Fakahany, 1999).

Signaling pathways for muscarinic receptors

Functionally, muscarinic receptors have been shown to activate various signaling pathways including phospholipase C, inhibition of adenylate cyclase, modulation of ion channels, activation of mitogen-activated protein kinase (involved in cell survival, differentiation and synaptic plasticity) and small GTPases (Volpicelli & Levey, 2004). Specifically, the M1, M3 and M5 receptors are coupled to G_q and G_{11} G proteins that leads to the activation of phospholipase C (Volpicelli & Levey, 2004). In addition M1, M3 and M5 receptors have also been shown to activate phospholipase A_2, phospholipase D and tyrosine kinases (Wess, 1996). In comparison, M2 and M4 receptors are coupled to G_i and G_o G protieins that leads to the inhibition of adenylate cyclase (Eglen, 2005). M2 and M4 receptors have also been shown to increase ATR receptor-induced phosphorylation of phospholipase A_2 (Wess, 1996), activate inward rectifying potassium channels and inhibit activation of fast voltage sensitive calcium channels (Hulme et al., 2003). All subtypes of muscarinic receptors have been shown to activate extracellular signal related kinase (ERK) (Hulme et al., 2003). Additionally, muscarinic receptors

have been shown to couple to G proteins other than the ones to which they preferentially couple. This may occur both in endogenous systems as well as in recombinant expression systems (Nahorski et al., 1997). Furthermore, the coupling to the various G proteins may be dependent on the agonist/antagonist bound (Nahorski et al., 1997). In general, when the receptor is activated, GDP is exchanged for GTP on the G protein resulting in its dissociation from the receptor (Wess, 1996). Subsequently, the G protein is dissociated into α and βγ subunits, which both activate downstream signaling cascades (Wess, 1996).

One pathway that is stimulated by receptor activation, primarily at the M1, M3 and M5 receptor subtypes, results in the production of inositol phosphates. In this pathway the α and/or βγ subunits of the G protein activates phospholipase C (PLC) (Caulfield, 1993; Lanzafame et al., 2003). Interestingly, there are thought to be at least nine isoforms of PLC, but the role of agonists in stimulating the various isoforms remains unclear (Caulfield, 1993). PLC then mediates the conversion of the membrane bound phosphatidylinositol 4,5-biphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Caulfield, 1993). IP_3 stimulates the release of calcium from intracellular stores within the endoplasmic reticulum (Caulfield, 1993; Felder, 1995). In one type of functional assay, the pool of PIP_2 is labeled with radiolabeled myo-inositol. Following stimulation with an agonist, different metabolites of myo-inositol are formed such as glycerophosphoinositol; myo-inositol 1-phosphate; 1, 4-bisphosphate; and inositol 1, 4, 5-triphosphate which can be quantitated (Berridge et al., 1983). The levels of each of the metabolites produced depend on the time period of exposure to the agonist (Berridge et al., 1983). Subsequently, these products are converted back into PIP_2 by phosphomonoesterases (Berridge et al., 1983). In parallel, DAG stimulates protein kinase C (PKC), although the levels of this stimulation appear to be minimal

when muscarinic receptor activation occurs (Caulfield, 1993). Stimulation of the effectors IP_3 and DAG result in alterations in cell transformation, differentiation and growth (Lanzafame et al., 2003).

Activation of phospholipase A_2 (PLA_2) as a result of inositol phosphate-induced calcium signaling through stimulation of the M1, M3 and M5 receptors has been shown to occur (Lanzafame et al., 2003). It is also thought that stimulation of the M2 and M4 receptors can result in indirect activation of PLA_2 (Lanzafame et al., 2003; Wess, 1996). PLA_2 stimulation results in the production of arachidonic acid and lysophospholipid as a result of phospholipid hydrolysis (Lanzafame et al., 2003). PLA_2 has been shown to play a role in membrane fluidity, and thus changes in PLA_2 activity may play a role in diseases such as Alzheimer's disease (Lanzafame et al., 2003).

Adenylate cyclase (AC) activity is modulated as a result of receptor stimulation by agonists. There have been shown to be at least ten isoforms of AC (Lanzafame et al., 2003), and the result of receptor activation may be dependent on which isoform of AC is present (Lanzafame et al., 2003). Generally, agonist binding to the M2 or M4 receptors results in inhibition of AC through the α subunit of the $G_{i/o}$ G proteins (Caulfield, 1993; Lanzafame et al., 2003). Subsequently, this results in a decrease in levels of cAMP (Caulfield, 1993). Furthermore, the decrease in cAMP results in a decrease in protein kinase A (PKA) activity. In contrast, it has been demonstrated that the M1, M3 and M5 receptors can also stimulate minimal levels of AC, resulting in an increase in cAMP levels and an increase in PKA activity (Caulfield, 1993; Felder, 1995). It is possible that the accumulation of cAMP following receptor activation could be a result of calcium or protein kinase C signaling (Felder, 1995; Nathanson, 2000).

Mitogen-activated protein kinase (MAPK) is divided into multiple pathways, one of which is extracellular signal regulated kinase (ERK) (Lanzafame et al., 2003). ERK

has been shown to influence cell growth, differentiation and survival (Werry et al., 2005). ERK signaling has been shown to greatly depend on protein-protein interactions. These interactions are important in allowing the signaling pathway to be complex; controling the order and orientation of substrates and enzymes; and targeting the signaling to specific subcellular locations (Werry et al., 2005). ERK activation is thought to occur as a result of agonist stimulation of all subtypes of muscarinic receptors (Lanzafame et al., 2003). When expressed in COS-7 cells both M1 and M2 receptors have been shown to stimulate ERK following exposure to carbachol (Crespo et al., 1994). In further support of the idea of the activation of ERK by agonists, stimulation of the M2 receptor has been shown to activate both Raf and Ras, which are upstream of ERK (Winitz et al., 1993). Thus, this activation occurs for both G_q and G_i G proteins. The activation of ERK through the M1, M3 and M5 muscarinic receptors is thought to be a result of PKC activation that is downstream of PLC activation (Lanzafame et al., 2003). In the case of M2 and M4 muscarinic receptors, activation of ERK appears to be dependent on the βγ subunit of the G protein (Lanzafame et al., 2003). The MAPK maximal response to agonist stimulation was observed between five and ten minutes; however, the response remained elevated for up to 90 minutes after agonist stimulation (Wotta et al., 1998). The activation of MAPK subsequent to stimulation of the receptor with an agonist is time and concentration dependent (Wotta et al., 1998).

There are thought to be six different G protein-coupled receptor kinases (GRKs) (Pitcher et al., 1998) that are involved in the desensitization of the response to agonist stimulation (Wess, 1996). They only phosphorylate G protein-coupled receptors when the receptors are activated by the binding of an agonist, which may have to do with the conformation of the receptor (Pitcher et al., 1998; Wess, 1996). Phosphorylation of the muscarinic receptors by GRKs is thought to occur on serine and threonine residues on

the third cytoplasmic loop of the receptor and possibly on the C-terminus (van Koppen & Kaiser, 2003). Phosphorylation by GRKs ultimately may result in sequestration of the receptor through the binding of arrestins (van Koppen & Kaiser, 2003; Wess, 1996). The phosphorylation by GRKs is thought to occur within minutes of the receptor being activated by an agonist (Pitcher et al., 1998). However, sequestration as a result of GRK phosphorylation does not appear to occur at all types of G protein-coupled receptors (Wess, 1996).

Consequences of prolonged exposure of muscarinic receptors to agonists and antagonists

Prolonged exposure of muscarinic receptors to agonists can result in receptor desensitization through mechanisms such as internalization, down-regulation, uncoupling of the G protein from the receptor and structural changes such as phosphorylation of the receptor (Maloteaux & Hermans, 1994). Initially receptor desensitization may occur without receptor internalization (Maloteaux & Hermans, 1994). When receptor internalization does occur cytoskeletal elements are thought to be important both for internalization and reinsertion of receptors into the cellular membrane (Maloteaux & Hermans, 1994). Acute desensitization results in a rapid decrease in the response with the return of the response occurring without new receptor synthesis (Dohlman et al., 1991). In contrast, long-term desensitization results in a decrease in receptor expression over time that requires protein synthesis to replace the deficit (Dohlman et al., 1991). This suggests that the decrease in muscarinic receptor expression occurs in two phases. The first is internalization in which the receptors can still be bound to the G protein and can subsequently be reinserted into the membrane. This is followed by down-regulation of the receptor (El-Fakahany & Cioffi, 1990).

Receptor expression can be measured with the aid of radiolabeled receptor antagonists. Two of these antagonists are $[^3H]$*N*-Methylscopolamine ($[^3H]$NMS) and $[^3H]$quinuclidinyl benzilate ($[^3H]$QNB). NMS is a permanently charged amine which can only bind to receptors expressed on the cell surface (Feigenbaum & El-Fakahany, 1985), whereas QNB is a lipophilic antagonist which can bind to receptors on the cell surface or internalized receptors that have not yet been reinserted or degraded (Ehlert et al., 1981; Vickroy & Malphurs, 1994).

When cells are treated with carbachol for prolonged periods of time there is a time dependent decrease in the levels of cell-surface receptor expression determined by both $[^3H]$NMS and $[^3H]$QNB binding; however, there was no change in the affinity of either radioligand (Cioffi & El-Fakahany, 1989; Hu et al., 1991). In contrast, brief treatment of cells expressing the M2 receptor with carbachol for 30 minutes resulted in a decrease in $[^3H]$NMS binding, but no change in $[^3H]$QNB binding, suggesting that the receptors are being internalized (Tsuga et al., 1998). This suggests that in binding studies, $[^3H]$NMS is more beneficial in detecting rapid changes in receptor expression on the cell surface compared to $[^3H]$QNB (Feigenbaum & El-Fakahany, 1984). When cells expressing M2 receptors were treated with carbachol, the maximal decrease in receptor expression resulted after 3 hours of treatment (May et al., 2005). In addition, Stope et al. (2003) demonstrated that treatment of cells expressing the M3 receptor with carbachol for 24 hours resulted in an approximately 50 percent reduction in the expression levels of the $G_{q/11}$ G proteins. When cells expressing the M1 receptor were exposed to carbachol, the G_q expression decreased to 50 percent of the maximum occurred even more rapidly (after 3 hours); however, in this case the treatment was found to have no effect on other G proteins (Mullaney & Milligan, 1993). A decrease in $G_{q/11}$ G protein expression independent of receptor down-regulation was also observed

by van de Westerlo et al (1995) in response to prolonged agonist treatment. Furthermore, work by Shifrin & Klein (1980) demonstrated that prolonged continuous activation or intermittent activation over time with carbachol results in a similar decrease in receptor expression on the cell surface. When atropine was used to block the orthosteric site during prolonged exposure to agonist there no longer was a decrease in receptor expression, demonstrating that the decrease in receptor expression was the result of agonist binding to the receptor (El-Fakahany & Cioffi, 1990). In contrast, the effect of prolonged pilocarpine, another muscarinic receptor agonist, treatment on receptor expression is less clear. Milligan & Strange (1983) found that there was no change in receptor expression following prolonged treatment with pilocarpine (6 hours) as measured by [^{3}H]NMS binding.

Agonist binding to the receptor can also influence the production of mRNA encoding the receptor. When CHO cells expressing M1 receptors were exposed to agonists, there was a reduction in mRNA that was associated with the agonist-induced down-regulation of the receptors (Wang et al., 1990). Additionally, the decrease in mRNA expression was shown to require a functional response because blocking the receptor with atropine during carbachol treatment resulted in only a minimal decrease in mRNA levels (Wang et al., 1990). Similarly, when cells expressing M3 receptors were exposed to carbachol for a prolonged period of time, there was a decrease in the expression levels of mRNA; however, the decrease occurred well after the decrease in cell-surface receptor expression (Fukamauchi et al., 1993; Steel & Buckley, 1993). Thus, the decrease in receptor expression levels following agonist treatment is most likely due to a combination of increased receptor degradation and a decrease in receptor production as a result of the decrease in mRNA expression (Wang et al., 1990).

There appears to be a correlation between agonist efficacy and the ability of the agonist to cause desensitization of the response (Hu et al., 1991). Furthermore, the desensitization of phosphoinositide (PI) hydrolysis appears to parallel the decrease in receptor expression (Thompson & Fisher, 1990). However, the time course of this response may depend on the conditions used. Coiffi & El-Fakahany (1989) found that the decrease in the ability of the agonist to stimulate inositol phosphate production occurred more slowly than down-regulation of cell-surface receptor density. For example, prolonged treatment of cells with the full agonist carbachol resulted in a time-dependent decrease in PI hydrolysis subsequent to further stimulation with carbachol (Hu et al., 1991; Mullaney & Milligan, 1993). Additionally, no increase in basal levels of PI hydrolysis was observed following carbachol treatment (Cioffi & El-Fakahany, 1989). In contrast, when cells were treated with carbachol followed by subsequent stimulation with the partial agonist McN-A-343, the decrease in response was more pronounced than that observed with carbachol (Hu et al., 1991). In addition, when McN-A-343 was used in place of carbachol in the pretreatment paradigm it was still able to desensitize its own response, but to a lesser degree than was observed with carbachol (Hu et al., 1991).

Research by van de Westerlo et al. (2005) was conducted to determine what regions of the receptor are important for receptor down-regulation in response to prolonged agonist stimulation. They demonstrated that when CHO cells expressing the M3 receptor were exposed to carbachol for prolonged periods of time the receptors were down-regulated; however, if a threonine to alanine mutation was made in the C-terminus of the receptor, down-regulation of the receptor no longer occurred. Additionally, although the threonine residues located at positions 550, 553 and 554 in the C-terminus appear to be important in receptor down-regulation they do not appear to play a role in

receptor binding or stimulation of signaling cascades (Yang et al., 1993). It is possible that these residues are sites for PKC phosphorylation; however, using an inhibitor to block phosphorylation in wild-type cells does not prevent receptor down-regulation (Yang et al., 1993).

Antagonists are not generally thought to induce receptor internalization (Maloteaux & Hermans, 1994). In contrast to treatment with agonists, long-term administration of atropine, a known muscarinic receptor antagonist, in rats (injections for seven days) resulted in an increase in [^{3}H]QNB binding in the cortex, hippocampus and heart (Lee & Wolfe, 1989). In addition, in a cell culture system, treatment of cells expressing the M2 receptor with antagonists, atropine (20 nM) or gallamine (20 μM), resulted in an increase in cell-surface receptor density with no change in radioligand affinity (May et al., 2005). Similarly, when cells expressing the M3 receptor were used, there was an increase in receptor expression following 24 hour exposure to atropine followed by washing (Ward et al., 2002). However, the increase in receptor expression occurred more slowly (over 12 hours) compared to the time needed for a decrease in receptor expression subsequent to prolonged treatment with an agonist such as carbachol (May et al., 2005). It should be noted that atropine was found to be an inverse agonist, rather than an antagonist, when studied using a constitutively active M3 receptor; thus, the effects on receptor density may be slightly different depending on the system (Thor et al., 2008). When prolonged *in vivo* treatment (injections for seven days) with another muscarinic receptor antagonist, scopolamine, was tested, it also resulted in an increase in cell-surface receptor density with no change in radioligand binding as measured by [^{3}H]QNB binding in tissues obtained from rat brain (Ben-Barak & Dudai, 1980). Together these results demonstrate that antagonist treatment can result in

upregulation of cell-surface receptor density, as opposed to agonist treatment down-regulating muscarinic cell-surface receptor density.

Influence of receptor mutations and expression levels on receptor activity

It should be noted that some G protein-coupled receptors have been shown to have endogenous activity in the absence of agonist stimulation. This activity can be a result of receptor mutations or changes in the expression level of the receptors. For example, some receptor mutations have been shown to result in high levels of constitutive receptor activity (Gether, 2000; Lefkowitz et al., 1993). Research has also demonstrated that mutations in the receptor can change the function of an antagonist to an agonist (Thor et al., 2008). Interestingly, although the amino acid sequence of muscarinic receptors appears to be conserved across species, minor differences in the amino acid sequence can result in dramatic changes in the pharmacological profile observed between different species (Caulfield & Birdsall, 1998).

Additionally, in *in vitro* receptor expression systems, high receptor expression can lead to elevated levels of basal receptor activation (Christopoulos & Kenakin, 2002; Milligan et al., 1995), possibly because more receptors will spontaneously be in the active state of the receptor (Chen et al., 2000). It has also been shown that partial agonists produce larger responses when there is high receptor expression or the receptor is more efficiently coupled to signaling cascades (Hu & El-Fakahany, 1990). Interestingly, various compounds have been shown to act as a partial agonist under one set of conditions but a full agonist under other conditions (Milligan et al., 1995). Thus, differences in responses can be observed for a given agonist depending on the level of receptor expression and the response system (Wang & El-Fakahany, 1993).

In vitro receptor expression systems

The use of receptor expression systems to study the properties of receptor binding and function can be beneficial. Some of the benefits of employing a cell culture system are that the cells are homogeneous, receptors can be expressed individually, cells are cultured in monolayer making it easier to administer drugs, it is possible to perform simultaneous experiments on receptor density and function and in intact cells, cellular localization of the receptor can be studied (El-Fakahany & Cioffi, 1990). The use of receptor expression systems is important because various tissues express multiple receptor subtypes, making it difficult to determine the properties of individual receptor subtypes (Bonner, 1989).

However, there are also potential problems with recombinant receptor expression systems. One problem is that in a recombinant expression system the receptor or the G protein may be interacting with membrane or cytosolic proteins that they do not endogenously associate with (Christopoulos & El-Fakahany, 1999; Kenakin, 1997). It is also possible that receptor overexpression in an experimental paradigm could lead to a compound being classified as a partial agonist in the experimental system, but when it is part a complex system it functions more like an antagonist (Christopoulos & El-Fakahany, 1999). Another potential problem is that the G protein levels may be low compared to the receptor expression levels, thus resulting in a lack of change of the receptor to a high affinity state which facilitates binding (Kenakin, 1997). It is possible that binding studies may be better determinants than functional studies because the result of binding studies will be an increased signal whereas in functional studies the maximal response can be saturated (Kenakin, 1997). Lastly, it may be possible to have selectivity *in vivo*, which is not observed *in vitro*, due to differences in the tissue such as affinity, efficacy, receptor number, and receptor-response coupling (Wood et al., 1999).

When using a receptor expression system it is also important to determine if the receptors of interest are expressed endogenously in the system. One cell line that is commonly used as an expression system for muscarinic receptors is Chinese hamster ovary (CHO) cells. When wild-type CHO cells were transfected with nitric oxide synthase there was a response to carbachol that was blocked by atropine, suggesting the presence of endogenous muscarinic receptors (Wang et al., 1995). However, muscarinic receptors were not detected with radioligand binding assays or functional assays looking at inositol phosphate production (Wang et al., 1995). Thus, this cell line is used to study muscarinic receptor biology, with the assumption that the endogenous expression of muscarinic receptors is negligible compared to the expression levels of the transfected muscarinic receptors.

Additionally, the experimental conditions of the assay need to be considered when using receptor expression systems, since differences have been observed between assays conducted with cells that are attached to a surface (monolayer) versus floating in buffer (suspension). For example, when forskolin-stimulated cAMP accumulation was studied there was a difference in the response when the assay was conducted in suspension versus monolayer. The assay conducted in suspension only resulted in 75 percent of the response obtained when cells were assayed in monolayer. In contrast, the response to pilocarpine was larger in cells in suspension compared to those assayed in monolayer (Mistry et al., 2005). This suggests that the response is dependent both on the agonist and the assay conditions. Additionally, when cells were treated with agonists and the level of receptor expression studied in monolayer or suspension there was a greater loss of cell-surface receptors for cells treated in monolayer compared to those in suspension as measured by [^{3}H]NMS binding (Thompson & Fisher, 1990). The difference between assays and the possible causes

for these observed differences are discussed further in Appendix A. In this thesis assays will be conducted in monolayer unless otherwise noted.

Allosteric interactions with the receptor

Allosteric sites have been found on a variety of receptors including ligand-gated ion channels, nicotinic receptors, $GABA_A$ receptors and G protein-coupled receptors (Gao & Jacobson, 2006). There are different types of allosteric modulators. Allosteric enhancers result in a conformation change at the orthosteric site that results in higher affinity for the ligand; allosteric agonists result in the G protein coupling more easily to the receptor irrespective of the action on the orthosteric site; and allosteric antagonists result in a decrease in receptor affinity and/or make the G protein less likely to couple to the receptor (Christopoulos & Kenakin, 2002; Wess, 2005).

Compounds that bind in an allosteric manner can result in conformational changes in the receptor which can result in the modulation of receptor activity (Christopoulos, 2002). In allosteric interactions, the binding of one ligand affects the ability of another ligand to bind. This occurs in a reciprocal manner between the two ligands (Birdsall et al., 1996). Changes in receptor conformation can result in changes in receptor affinity. Positive cooperativity results in an increase in affinity, negative cooperativity results in a decrease in affinity and neutral cooperativity results in no change in affinity (Christopoulos, 2002). The action of a particular allosteric modulator may be ligand specific. For example, WIN51708 displayed positive cooperativity with $[^3H]$NMS binding, however it had negative cooperativity with $[^3H]$QNB binding (Lanzafame et al., 2006). Thus, it may be possible to get some degree of subtype selectivity based on cooperativity rather than affinity (Lanzafame et al., 2006). In addition, it is important to consider that the effect on receptor function as a result of

allosteric modulation is not always the same as it is on binding (Christopoulos, 2002). It is also important to consider that the binding of allosteric ligands to the receptor can be dependent on the experimental conditions such as ionic strength, divalent cations and temperature (Birdsall & Lazareno, 2005).

Interaction of a compound through an allosteric site may be more advantageous than acting at the orthosteric site because it may be possible to get greater subtype selectivity since allosteric sites likely have greater sequence divergence across receptor subtypes. If the effect of the allosteric modulator is saturable it could prevent problems with overstimulation. Additionally, the activation of the response could be manipulated so the response occurs only when the endogenous agonist is present resulting in tissue specific activation (Christopoulos, 2002; Christopoulos & Kenakin, 2002).

Pharmacological profile of allosteric binding

Allosteric modulation of receptors results in changes in the binding and functional properties determined using various receptor assays. One classic hallmark of allosteric modulation is the inability to decrease binding to non-specific levels when employing an inhibition of radioligand binding assay. This is dependent on the concentration of the radioligand (Christopoulos & Kenakin, 2002; Lee & El-Fakahany, 1991). Additionally, using a saturation binding paradigm, the shift in binding is saturable with increasing concentrations of the allosteric agent (Christopoulos & Kenakin, 2002). Allosteric binding also has the ability to change the association and/or dissociation kinetics of a reaction at the orthosteric site, which can change the time that it takes the system to reach equilibrium (Birdsall & Lazareno, 2005; Christopoulos, 2002; Christopoulos & Kenakin, 2002). In radioligand binding assays positive cooperativity is determined using

a radioligand concentration below the K_d, whereas negative cooperativity is determined using a high concentration of radioligand (Birdsall & Lazareno, 2005).

Allosteric sites on muscarinic receptors

It is possible that there are multiple allosteric binding sites on muscarinic receptors (Christopoulos & Kenakin, 2002; Wess, 2005). It is also possible that a single receptor can have multiple allosteric modulators bound at different sites at the same time (Christopoulos & Kenakin, 2002). It has been suggested that a possible allosteric site on muscarinic receptors is located near the orthosteric site, but more extracellularly (Christopoulos & Kenakin, 2002). It is also likely that muscarinic receptors have specific allosteric sites because some allosteric modulators are able to compete with each other (Christopoulos & Kenakin, 2002). These allosteric sites may overlap across receptor subtypes or have varying degrees of subtype selectivity.

A variety of compounds have been found to be allosteric modulators of muscarinic receptors. These include compounds such as neuromuscular blockers, reactivators of cholinesterases, ion channel blockers, and local anaesthetics, among others (Tucek & Proska, 1995). One such compound is gallamine. Gallamine is thought to be an allosteric modulator at all subtypes of muscarinic receptors (Clark & Mitchelson, 1976; Wess, 1996). However, the effects appear to be the most pronounced at the M2 receptor subtype (Wess, 1996). It was able to inhibit responses to both acetylcholine and carbachol in the guinea pig atria (Clark & Mitchelson, 1976). Gallamine decreased both the association and dissociation of additional ligands at the orthosteric binding site (Tucek & Proska, 1995; Wess, 1996). Gallamine was not able to completely inhibit [^{3}H]NMS binding, which is a hallmark of an allosteric modulator (Spalding et al., 2006). In a saturation binding paradigm, gallamine reduced [^{3}H]NMS binding affinity in a

manner that was distinct from a conventional reversible agonist (Lee & El-Fakahany, 1991). Additionally, gallamine may mediate changes in the receptor conformation resulting in positive cooperativity with the G protein, but negative cooperativity with the agonist binding site (Christopoulos & Kenakin, 2002). It is thought that gallamine binds at an extracellular location on the receptor, perhaps extracellularly to the agonist binding site in a manner in which it is able to at least partially block the orthosteric site (Birdsall et al., 1996).

Alcuronium is another allosteric modulator of muscarinic receptors. It has been shown to inhibit the dissociation of [^{3}H]QNB from the receptor and inhibit both the association and dissociation of [^{3}H]NMS from the receptor (Tucek & Proska, 1995). However, its effects on binding of [^{3}H]NMS are dependent on the tissues being studied. Alcuronium resulted in an increase in radioligand affinity in rat heart, ileum and cerebellum, which are tissues that are thought to express primarily M2 and M4 receptors, but a decrease in affinity in the cerebral cortex and salivary glands which are thought to express primarily M1, M3 and M5 receptors (Tucek & Proska, 1995). Similar to gallamine, alcuronium may bind to a region on the receptor that blocks further access to the orthosteric site either directly or through steric modifications (Tucek & Proska, 1995). One possible explanation for the discrepancy observed between the binding kinetics versus the affinity of alcuronium is that allosteric binding in this case results in changes in the affinity of the receptor that may be due specifically to conformational changes, while a change in kinetics may be the result of steric hindrance (Tucek & Proska, 1995).

Strychnine acts as an allosteric modulator of muscarinic receptors. For example, at the M4 receptor it has been shown to inhibit acetylcholine binding, but enhance [^{3}H]NMS binding (Birdsall et al., 1996). Additionally, strychnine has been shown to

decrease [^{3}H]NMS dissociation at the M4 receptor (Birdsall et al., 1996). Strychnine has also been shown to change the kinetics at the other receptor subtypes (Birdsall et al., 1996).

AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride) is an example of a novel muscarinic agonist that is thought to have functional activity at only the M1 receptor subtype (Spalding et al., 2002). However, it was able to inhibit [^{3}H]NMS binding at all subtypes of muscarinic receptors with a low potency (Spalding et al., 2002). Interestingly, the activation of the M1 receptor by AC-42 occurs at a region other than that used by carbachol, demonstrating that it is binding in an allosteric manner (Spalding et al., 2002). Mutation data for regions in the third transmembrane domain suggest that AC-42 may bind closer to the extracellular space, whereas carbachol binds deeper in the transmembrane domain of the receptor (Spalding et al., 2006). Furthermore, the N-terminus of the receptor was shown to be necessary to obtain a functional response to AC-42 (Spalding et al., 2002).

Recent research has resulted in further development of allosteric modulators of muscarinic receptors with subtype selectivity. One such drug is 1-(1’-2-methylbenzyl)-1,4’-bipiperidin-4-yl)-1H benzo[d]imidazol-2(3H)-one (TBPB) (Jones et al., 2008). It is structurally different than any of the other allosteric modulators that are currently known. TBPB has been shown to selectively activate the M1 receptor in an allosteric manner (Jones et al., 2008). When cells were treated with increasing concentrations of atropine there was a decrease in the ability of TBPB to stimulate a response, as well as a rightward shift in the functional curve, which is an indication of allosteric modulation (Jones et al., 2008). Interestingly, TBPB has been shown to potentiate NMDA currents in the hippocampus which is thought to be mediated by M1 receptor activation (Jones et

al., 2008). However, TBPB had some antagonist activity at the D2 dopamine receptor, which complicates any potential *in vivo* application (Jones et al., 2008).

Another recent report presented data on the development of a set of compounds that are positive allosteric modulators at the M4 receptor. In functional assays these compounds had no activity on their own but potentiated the response to acetylcholine at the M4 receptor (Shirey et al., 2008). However, there was no response alone or in conjunction with additional agonist to these compounds at any of the other muscarinic receptor subtypes (Shirey et al., 2008). It appears that these compounds may increase the affinity of the orthosteric site to acetylcholine and/or though positive cooperativity with the G protein (Shirey et al., 2008).

Muscarinic receptors, allosteric sites and diseases

Muscarinic acetylcholine receptors are thought to play a role in a variety of diseases of the central nervous system such as Alzheimer's disease, Parkinson's disease, depression, epilepsy and schizophrenia (Wess, 2004). The potential role of muscarinic receptors in Alzheimer's disease and schizophrenia will be discussed below. The possible use of a compound that acts as an allosteric modulator of muscarinic receptor will be discussed as a possible treatment for these diseases.

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease that leads to a progressive loss of cognitive function caused by neuronal loss in the hippocampus and various neocortical regions (Keller, 2006; Kotilinek et al., 2002). It is the most common cause of dementia in the elderly with the clinical characteristics of AD involving changes in behavior and personality (Minghetti, 2005; Reddy, 2006). It has been estimated that

approximately four million people may currently have AD (Campbell, 2004), a number that is projected to increase to nearly 13.5 million by 2050 (Hodes, 2006). AD is the eighth leading cause of death in the United States (Hodes, 2006). Although the exact cause of AD is unknown, the majority of AD is thought to be a result of sporadic mutations, with only two percent of cases attributed to a familial form (Reddy, 2006).

Although currently AD can only definitively be diagnosed post-mortem, it is clinically diagnosed based on clinical and neuropsychological exams and by the exclusion of other forms of dementia (Maccioni et al., 2004). Prior to being diagnosed with AD, patients have mild cognitive impairment (MCI), during which some beta amyloid plaques and neurofibulary tangles are present. Interestingly, not all patients that have MCI progress to AD (Ashe, 2005; Blennow, 2004). The most common symptoms of AD include a decline in memory, language, visuospatial orientation, and possibly depression (Maccioni et al., 2004). Additionally, the loss of olfaction may occur before the cognitive and behavioral decline in patients with AD (Suzuki et al., 2004).

Although the exact cause of AD remains unclear, there are multiple changes or deficits observed in postmortem analysis of the brains of Alzheimer's disease patients such as the loss of cholinergic neurons (Francis et al., 1999), accumulation of β-amyloid plaques (Hardy et al., 1998), formation of tangles of hyperphosphorylated tau (Hardy et al., 1998), changes in various receptor expression (muscarinic receptors, Flynn et al., 1995; nicotinic receptors, Messer, 2002) and others.

Cholinergic hypothesis of Alzheimer's disease

One characteristic of AD is the loss of cholinergic neurons in the basal forebrain that project to the hippocampus (Francis et al., 1999). Acetylcholine is synthesized from acetyl-CoA and choline by the enzyme choline acetyltransferase (Raedler et al., 2007).

Acetylcholine is created in the neurons and stored in presynaptic vesicles before release (Raedler et al., 2007). In AD there is a decrease in the levels of choline acetyltransferase which appears to correlate with the degree of cognitive impairment (Francis et al., 1999). The regions that seem to be the most affected by cholinergic degeneration are pyramidal neurons, nucleus basalis of Meynert, medial septum, raphe nuclei and the locus coeruleus (Francis et al., 1999). At the end-stages of AD there may be up to a 90 percent loss of cholinergic neurons that project from the basal forebrain to cortical and hippocampal areas (Yan & Feng, 2004). Interestingly, in terms of receptor expression, the presynaptic M2 receptors are lost (Flynn et al., 1995; Francis et al., 1999); however, the postsynaptic M1 and M3 receptors appear to remain at normal levels (Eglen, 2005; Flynn et al., 1995). Additionally, the levels of presynaptic M4 receptors were increased whereas the level of postsynaptic M5 receptors remained unchanged (Flynn et al., 1995). Although the number of M1 receptors remains the same between people with AD and control subjects, the binding affinity may not be the same (Flynn et al., 1991). Interestingly, the loss of the ability of muscarinic cholinergic agonists to bind occurs in brain regions that are associated with changes in AD, but not in unassociated brain regions (Flynn et al., 1995). For example, Flynn et al. (1991) demonstrated that the ability of carbachol to bind to M1 receptors with high-affinity in the cortex was reduced in AD; however, the low affinity binding remained unchanged. This change may be a result of changes in M1 receptor-effector coupling (Flynn et al., 1991). Additionally, the potential change in function of the M1 receptors in AD may be at least part of the reason why cholinergic therapies are not always effective treatments for AD (Flynn et al., 1991).

Cholinergic treatments for Alzheimer's disease

In AD the impaired cholinergic neurotransmission is thought to result in deficits in memory and cognition (Zimmermann et al., 2005). The degeneration of presynaptic cholinergic neurons results in a decrease in the levels of acetylcholine present in the synapse, which further results in a decrease in the stimulation of the postsynaptic neuron (Oda, 1999). However, the postsynaptic receptors are thought to be relatively intact (Flynn et al., 1995). The first treatment strategy that was employed was to inhibit the breakdown of acetylcholine into acetate and choline by acetylcholinesterases in order to increase levels of acetylcholine in the synapse (Mesulam, 2004). Thus, the compounds that were initially developed and used in the treatment of Alzheimer's disease were acetylcholine esterase inhibitors (AChEI's) such as tacrine (Cognex), donepezil (Aricept), rivastigmaine (Exelon) and galanthamine (Reminyl) (Disterhoft & Oh, 2006). One potential problem with this type of treatment is that it relies on release of acetylcholine from presynaptic nerve terminals, which is an issue because the presynaptic terminals have been shown to degenerate (Francis et al., 1999). Additionally, the higher levels of acetylcholine in the synapse not only stimulate the M1 receptors which are the desired target but also other muscarinic receptors subtypes which are thought to mediate the side-effects of treatments such as nausea, vomiting, fainting, weight loss, muscle fatigue and bradycardia (Bianchetti et al., 2006). A recent clinical trial suggested that transdermal application of these types of drugs may result in amelioration of the cognitive and behavioral symptoms of AD without some of the side effects observed with oral administration. Curently, the reason for the decreas in side effects following transdermal drug application is unclear. However, more research is needed before this could become a wide spread treatment option (Chan (a) et al., 2008).

The second type of strategy that was employed to treat AD was the use of cholinergic agonists to replace the deficiency in endogenous acetylcholine. Such agonists would be beneficial because they do not rely on presynaptic cholinergic projections to be present (Bymaster et al., 1997). However, it is necessary to find an agonist that selectively activates the M1 receptor to avoid unwanted side effects. The creation of various M1 selective agonist compounds has been attempted. These include compounds such as AF102B, AF150, talsaclidine and xanomeline (Fisher et al., 1996; Messer, 2002). Treatment with these compounds resulted in cognitive improvements measured by the AD assessment scale; however, cholinergic side effects were seen similar to those observed with acetylcholinesterase inhibitors treatment making them impractical as clinically relevant treatments for AD (Fisher et al., 1996). The results of treatment with xanomeline will be discussed in more detail below. However, it is possible that a new therapeutic could be developed in which there is greater subtype selectivity or there is positive cooperativity between the drug and acetylcholine which would facilitate the endogenous response (Jakubik et al., 1997). A drug with positive cooperativity would allow a tissue specific response and decrease the side effects normally observed with treatment (Jakubik et al., 1997).

Non-cholinergic treatments for Alzheimer's disease

AD results from a compilation of many different factors. Thus, additional treatments have been tried for AD that target potential aspects of the disease that are not directly related to cholinergic transmission. There is some evidence for increases in oxidative species or inflammation, thus, treatment strategies have included antioxidant and anti-inflammatory therapy (Zimmermann et al., 2005). Additionally, a particular form of the apolipoprotein E, the type 4 allele, is thought to play a role in AD (Corder et al.,

1993). Treatment with statins were used to try to address this problem (Zimmermann et al., 2005). Another treatment is the use of an NMDA receptor antagonist, memantime (Namenda), which inhibits calcium from entering neurons (Disterhoft & Oh, 2006). This effect may be independent or in accordance with changes in cholinergic transmission. Furthermore, the accumulation of non-soluble β-amyloid peptides in AD has been shown to be neurotoxic. A relatively new treatment strategy employs the use of a vaccine against β-amyloid with the goal being to decrease the levels of β-amyloid deposits in the brain (Wisniewski & Konietzko, 2008). This vaccine showed great promise in mouse models of AD. However, it had mixed results when administered to human subjects; some patients developed an immune response that decreased β-amyloid deposition whereas others had no apparent immune response (Wisniewski & Konietzko, 2008). Although multiple approaches for the treatment of AD may be beneficial these types of treatments based on non-cholinergic targets have not been approved for clinical use and will not be further addressed here.

Schizophrenia

Schizophrenia is a mental illness that is generally characterized by behavioral symptoms such as psychosis, apathy, social withdrawal and disorganized speech (Mueser & McGurk, 2004; Raedler et al., 2007). Typically people with schizophrenia have delusions and hallucinations (Mueser & McGurk, 2004). These are thought to be due to hyperactivity of dopamine signaling in the brain (Bymaster et al., 2002). In addition, schizophrenics also have cognitive deficits in learning and memory, problem solving and problems with attention (Mueser & McGurk, 2004). There are thought to be both genetic and environmental components that lead to schizophrenia (Mueser & McGurk, 2004).

Schizophrenia can be diagnosed using different types of imaging. Schizophrenic patients have larger ventricles compared to unaffected people (Mueser & McGurk, 2004). Positron emission tomography has shown that there is a change in blood flow to the thalamus and cerebellum (Mueser & McGurk, 2004). Additionally, functional magnetic resonance imaging suggests that there are global changes in brain activity rather than changes that are specifically localized to particular brain regions (Mueser & McGurk, 2004). These changes may be a result of changes that occurred during development, resulting in abnormal connectivity in the brain (Mueser & McGurk, 2004).

It is thought that problems with dopamine signaling are involved in schizophrenia. When the D2 dopamine receptor is antagonized, there is a decrease in symptoms such as delusions and hallucinations (Raedler et al., 2007). To further support this idea, imaging studies conducted on schizophrenics have demonstrated that there is an increase in dopamine neurotransmission in their brains (Raedler et al., 2007). However, changes in dopamine transmission can not account for all of the symptoms of schizophrenia; thus, it has been proposed that other neurotransmitter systems may also play a role (Raedler et al., 2007).

Studies have shown that there is an interaction between the dopaminergic and cholinergic systems in the brain, although the specifics of the interactions are still unclear (Raedler et al., 2007). In schizophrenic patients, a reduction in the numbers of muscarinic and nicotinic acetylcholine receptors have been found in the cortex and hippocampus (Friedman, 2004). Additionally, there is some evidence that there is also a decrease in muscarinic receptors in the ventral striatum (Raedler et al., 2007). These decreases are thought to be primarily in the M1 and M4 receptor subtypes (Raedler et al., 2007).

Treatment for schizophrenia

Schizophrenia is frequently treated pharmacologically with antipsychotics. However, these drugs can have negative side-effects in terms of cognition, extrapyramidal symptoms and tardive dyskinesia (Mueser & McGurk, 2004). There are two classes of drugs that are frequently used in treatment, typical and atypical antipsychotics (Bymaster et al., 2002). The most commonly used typical antipsychotics are haloperidol and chlorpromazine, which are dopamine antagonists (Bymaster et al., 2002). In contrast, the atypical antipsychotics are thought to be serotonin/dopamine antagonists and include drugs such as risperidone and clozapine (Bymaster et al., 2002). Treatments for schizophrenia also include antidepressants and benzodiazepines (Mueser & McGurk, 2004). However, many schizophrenic patients fail to take their medication due to the adverse side effects (Mueser & McGurk, 2004). In some cases, psychological treatment is also tried in addition to pharmacological treatments (Mueser & McGurk, 2004).

New treatments for schizophrenia are being employed using cholinergic agonists because the cholinergic system is known to modulate the dopaminergic system (Bymaster et al., 2002). Cholinergic agonists have been shown to have antipsychotic effects in schizophrenics (Andersen et al., 2003). In some cases, cholinesterase inhibitors have been administered in conjunction with the traditional antipsychotics with mixed results. Some schizophrenics see an improvement in cognitive symptoms whereas others see no apparent benefit (Raedler et al., 2007).

Binding and functional properties of xanomeline

Xanomeline (3-(4-hexyloxy-1,2,5-thiadiazol-3-yl)-1,2,5,6-tetrahydro-1methylpyridine) was developed by Eli Lily as a potential therapeutic agent for the

treatment of AD (Sramek et al., 1995). Xanomeline is based on an arecoline derivative that is found naturally as the active ingredient in betel nuts (Raedler et al., 2007). In the initial analysis of xanomeline, it was found to have a high affinity for muscarinic receptors but lacked affinity at other non-muscarinic types of receptors (Bymaster et al., 1997; Mirza et al., 2003).

Xanomeline is a potent agonist that is able to bind to all subtypes of muscarinic receptors with similar potency, but is thought to be functionally selective for the M1 and M4 subtypes (Bymaster et al., 1997; Shannon et al., 1994). Functionally, xanomeline is a potent full agonist at the M1 receptor. In contrast, it is a potent partial agonist at the M2 and M5 receptors (Grant & El-Fakahany, 2005; Jakubik et al., 2006). Xanomeline has been shown to stimulate a functional response at the M1 receptor using multiple types of functional assays such as PI hydrolysis, citrulline formation and ERK phosphorylation in CHO cells (Christopoulos et al., 1999; Christopoulos et al., 1998) (Christopoulos, personal communication). At the M1 and M2 receptors, xanomeline exhibits selectivity in the coupling of the receptor to specific G proteins (Jakubik et al., 2006). Contradictory research on the functional properties of xanomeline conducted by Wood et al. (1999) determined that xanomeline was a full agonist at the M3 and M4 receptors, and a partial agonist at M1, M2 and M5 receptors. One possible explanation for the difference may be that there was receptor reserve at the M3 receptor, which made xanomeline appear to be a full agonist under these conditions (Wood et al., 1999). Additionally, they were using microphysiometry which detects changes in extracellular acidification as a measure of receptor activation (Wood et al., 1999).

Xanomeline has been shown to have a unique binding profile. Research conducted by Christopoulos et al (1998) at the M1 receptor demonstrated that there was not simple competition between xanomeline and atropine since the slope of the

functional PI assay curve was altered rather than simply shifted to the right as was observed with carbachol and atropine. Additionally, brief treatment with xanomeline resulted in a decrease in radioligand affinity in saturation binding experiments, also indicative of a non-competitive interaction (Christopoulos et al., 1998). Xanomeline was also shown to bind to the M1 receptor in a manner that was resistant to washout and was dependent on exposure time (Christopoulos et al., 1998). When xanomeline and atropine were present concurrently, atropine was able to block a functional response. However, when atropine was washed out xanomeline was again able to stimulate a functional response at the M1 receptor, suggesting some type of competitive interaction at the orthosteric site (Christopoulos et al., 1998). Furthermore, the presence of xanomeline in a competition binding assay with [^{3}H]NMS, resulted in complete inhibition of [^{3}H]NMS which is thought to be a result of competitive interactions with the receptor orthosteric site (Christopoulos et al., 1998). Additionally, there is also evidence that xanomeline interacts with the M1 receptor orthosteric site through the transmembrane domain of the receptor in a similar manner to that of carbachol, since the mutation of threonine 192 to alanine, results in a decrease in agonist affinity for both drugs compared to the affinity for wild-type receptors (Tejada et al., 2006). Thus it appears that xanomeline interacts with the receptor in both a competitive and non-competitive manner (Christopoulos et al., 1998).

Proposed mechanisms of xanomeline binding

Based on the previous findings, a model for xanomeline binding at the M1 receptor was proposed. Binding of xanomeline at the M1 receptors occurs at both the orthosteric (primary) site (reversible binding) and an allosteric (secondary) site (wash-resistant binding) (Christopoulos et al., 1998). However, it is unclear if binding of

xanomeline occurs through the same mechanisms at other receptor subtypes. The model of xanomeline binding proposed by Christopoulos et al. (1998) was based on the interaction of the β_2-adrenoceptor agonist salmeterol studied by Coleman et al (1996) and Nials et al (1993).

Wash-resistant xanomeline binding

Current laboratory research has primarily focused on the effects of xanomeline binding at the M1 receptor. Binding of xanomeline in a wash-resistant manner at the M1 receptor occurs rapidly (Jakubik et al., 2002). Wash-resistant binding also occurs at the M2 receptor, but the kinetics are slower than at the M1 (Jakubik et al., 2006). Additionally, research by Grant & El-Fakahany (2005) demonstrated that xanomeline is also able to bind in a wash-resistant manner at the M5 receptor. The binding of xanomeline to the M1 receptor occurs even if the primary binding site is blocked (Jakubik et al., 2002). The dissociation of the wash-resistant component of xanomeline binding from the M1 receptor occurs very slowly with a half life of over 30 hours (Jakubik et al., 2002). In addition, incubation of cells that express the M1 muscarinic receptor with xanomeline followed by washing leads to an apparent decrease in the number of binding sites as determined by saturation binding (Jakubik et al., 2002).

The alkyloxyl side chain of xanomeline is necessary for the occurrence of wash-resistant binding to the M1 receptor (Jakubik et al., 2004). When the hydrophobic tail of xanomeline (hexyl, 6 carbons) is reduced to propyl (3 carbons) or methyl (1 carbon), the xanomeline analog no longer is able to bind in a wash-resistant manner at the M1 receptor (Jakubik et al., 2004; Kane et al., 2008). Furthermore, the xanomeline analogs with shortened side-chains that were still able to bind in a wash-resistant manner (butyl, 4 carbons and pentyl, 5 carbons) were able to interfere with wash-resistant binding of

xanomeline demonstrating that the analogs are binding to the receptor in a manner similar to that of xanomeline (Jakubik et al., 2004). Interestingly, although both the butyl and pentyl xanomeline analogs were able to bind in a wash-resistant manner only the pentyl analog was still able to stimulate a functional response in a wash-resistant manner (Jakubik et al., 2004).

Additional experiments were conducted to determine if xanomeline binds specifically in a wash-resistant manner to the receptor or if it simply partitions in a nonspecific manner into the lipid membrane. Xanomeline was able to bind in a wash-resistant manner when purified M1 receptors were reconstituted in liposomes, but not when receptors or liposomes were exposed to xanomeline alone prior to reconstitution. This demonstrates that xanomeline binds to the M1 receptor in a wash-resistant manner; however, the receptor needs to be in a lipid environment for this binding to take place (Jakubik et al., 2004). The importance of a lipid environment can also be seen by lowering the pretreatment temperature to 4 or 15°C which resulted in a decrease in affinity of wash-resistant xanomeline binding (Jakubik et al., 2004).

Jakubik et al (2004) suggested that if the underlying mechanism of wash-resistant binding of xanomeline is elucidated it may lead to the development of both muscarinic agonists and antagonists with long duration of action and perhaps better pharmacological selectivity. In order to determine the mechanism of xanomeline binding it is important to investigate the action of xanomeline across subtypes using the same receptor expression system.

Xanomeline as a treatment for Alzheimer's disease and schizophrenia

Although the mechanisms for Alzheimer's disease and schizophrenia are likely different, there is an overlap in some of the symptoms, such as delusions, hallucinations,

memory deficits, etc., which can be alleviated by treatment with cholinergic agonists (Mirza et al., 2003). Thus, research has been conducted using xanomeline as a potential therapeutic for both diseases in animal models and in clinical studies.

Xanomeline treatment in Alzheimer's disease models

The characteristics of xanomeline binding and function were studied in animal models. Xanomeline was found to bind with high affinity in rat hippocampus and rabbit vas deferens, both of which are thought to primarily express M1 receptors (Shannon et al., 1994). In contrast, minimal binding of xanomeline was observed in guinea pig atria (M2 receptors) or guinea pig urinary bladder (M3 receptors) (Shannon et al., 1994). Treatment with xanomeline resulted in an increase in striatal levels of a metabolite of dopamine and an increase in the levels of acetylcholine in the rat, suggesting that both dopamine and acetylcholine release were elevated in response to xanomeline. Since there is known to be an interaction between dopamine and muscarinic receptors and xanomeline has not been shown to interact with dopamine receptors, it is thought that the response is mediated by M1 receptors (Bymaster et al., 1994). Interestingly, the effects of xanomeline on these neurotransmitters in other brain regions were variable (Bymaster et al., 1994). When rats were treated with xanomeline, an increase in salivation, which is thought to be mediated by the M3 receptor, was only detected in one of five animals at a high dose (Bymaster et al., 1994). When tissue slices of rat brain were treated with xanomeline *in vitro* the release of acetylcholine was inhibited following additional stimulation, but the basal release was unchanged (Machova et al., 2007). Furthermore, when tissues were preincubated with xanomeline there was a concentration-dependent decrease in acetylcholine release following carbachol-induced stimulation (Machova et al., 2007). These results are most likely due to the activation of

the M2 and/or M4 muscarinic receptors. Taken together these results suggest that the actions of xanomeline may be efficacious in the treatment of AD.

Clinical trials with xanomeline for the treatment of Alzheimer's disease

Clinical trials were conducted to determine the effects of xanomeline treatment in AD. Initial data demonstrated that xanomeline was able to cross the blood brain barrier, and thus would be able to reach the targeted M1 receptors (Bymaster et al., 1997). Interestingly, the concentration of xanomeline in the brain was found to be ten to twenty times higher compared to plasma levels (Mirza et al., 2003). Xanomeline was also shown to undergo high first past metabolism, which occurs through β-oxidation of the aliphatic side chain and N-oxidation of the tetrahydropyridine ring (Bymaster et al., 1997). The N-desmethyl metabolites have been shown to be pharmacologically active (Mirza et al., 2003).

Xanomeline was able to reduce cognitive deficits and behavioral symptoms in Alzheimer's disease patients, which are thought to be due to activation of the M1 receptor (Bymaster et al., 1997). This occurred in patients that had both mild and moderate AD (Bymaster et al., 1997). The maximum effect on cognition occurred between eight and ten weeks after the start of drug administration (Bodick et al., 1997 (b); Bodick et al., 1997 (a)). In addition to improvements in cognitive deficits, treatment of AD patients with xanomeline resulted in a reduction in outbursts, suspiciousness, delusions, agitation and hallucinations (Bodick et al., 1997 (b); Mirza et al., 2003). However, treatment with xanomeline also resulted in adverse side effects such as hypersalivation, bradycardia, gastrointestinal tract problems, sweating, nausea, vomiting, and syncope, which are thought to be mediated mainly by non-selective activation of the M2 and M3 muscarinic receptors (Bodick et al., 1997 (b); Bymaster et al., 1997). In a

clinical trial conducted by Sramek et al. (1995) 94 percent of patients experienced some side effects. Interestingly, higher plasma concentrations of xanomeline appeared to correlate with more adverse effects (Sramek et al., 1995). The side effects of xanomeline may be due to non-selective receptor activation by xanomeline and/or as a result of activation by metabolites from the metabolism of the alkyloxy side chain of xanomeline (Messer, 2002). Additionally, it may be possible that xanomeline has different effects in different areas in the brain (Mirza et al., 2003).

Xanomeline treatment in animal models of schizophrenia

Startle amplitude, locomotor activity, prepulse inhibition and conditioned avoidance are used as behavioral methods to assess schizophrenia in animal models (Bymaster et al., 2002). The administration of xanomeline resulted in a decrease in startle amplitude in a dose-dependent manner (Jones et al., 2005; Stanhope et al., 2001). Treatment with a non-selective muscarinic agonist, pilocarpine, was shown to have a similar effect compared to xanomeline on decreasing startle amplitude, demonstrating that the decrease in response is mediated by muscarinic receptors (Stanhope et al., 2001). In animals, treatment with amphetamine has been shown to increase locomotion as a result of an increase in dopamine in the synapse (Bymaster et al., 2002). Administration of xanomeline was shown to decrease amphetamine-induced hyperlocomotion, while having no effect on baseline locomotion (Bymaster et al., 2002; Stanhope et al., 2001). Schizophrenics have been shown to have a decrease in prepulse inhibition of the acoustic startle reflex (Raedler et al., 2007). In prepulse inhibition, a nonstartling tone that occurs just prior to an intense stimulus reduces the startle response (Bymaster et al., 2002). Treatment with xanomeline is able to reverse this deficit in an animal model of schizophrenia (Raedler et al., 2007; Stanhope et al.,

2001). Taken together these animal models suggest that xanomeline may alleviate some of the symptoms of schizophrenia in humans. The antipsychotic profile of xanomeline may be mediated through the M1 or M4 muscarinic receptor, which at this time remains unclear (Mirza et al., 2003).

Clinical trials with xanomeline for the treatment of schizophrenia

A recent clinical trial was conducted employing xanomeline as a treatment for schizophrenia. Treatment of schizophrenic patients with xanomeline resulted in improvements in total Positive and Negative Syndrome Scale scores, verbal learning and short-term memory function by the end of the first week of a four week treatment period (Shekhar et al., 2008). There were minimal adverse side effects such as gastrointestinal tract problems, but they were not severe enough to discontinue treatment (Shekhar et al., 2008). Thus, preliminary results suggest that xanomeline may be beneficial in the treatment of schizophrenia.

Summary

Muscarinic receptors (M1-M5) are G protein coupled receptors that are located widely throughout the brain and periphery. These receptors are known to be involved in a variety of diseases, such as Alzheimer's disease and schizophrenia, which has made them targets for drug development. Currently, subtype selective agonists and antagonists that bind at the orthosteric site are not available due to the highly conserved nature of the orthosteric site. New research is beginning to focus on allosteric sites as drug targets because these sites are thought to be less conserved across subtypes, thus potentially resulting in greater subtype selectivity. Xanomeline is a novel muscarinic receptor agonist that binds at both the orthosteric site and at an allosteric site. This

thesis will address the effects of acute and long-term xanomeline treatment on muscarinic receptors in terms of binding and functional properties.

CHAPTER 2: Materials and methods

MATERIALS AND GENERAL TREATMENT PROTOCOLS

Materials

$[^3H]$*N*-Methylscopolamine ($[^3H]$NMS) (81 Ci/mmol) was purchased from DuPont (Wilmington, DE); $[^3H]$quinuclidinyl benzilate ($[^3H]$QNB) (52 Ci/mmol) was purchased from DuPont (Wilmington, DE); *myo*-$[^3H]$inositol (71 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK); $[^{14}C]$inositol-1-phosphate (300 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO); Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA); geneticin was obtained from Calbiochem (San Diego, CA); siRNA was purchased from Ambion (Austin, TX); and bovine calf serum was supplied by Hyclone Laboratories (Logan, UT). Xanomeline tartrate was a generous gift from Eli Lilly & Co. (Indianapolis, IN); all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Chinese hamster ovary (CHO) cells stably transfected with the human M1-M4 muscarinic acetylcholine receptors (hM1-hM4) were provided by Dr. M. Brann, University of Vermont Medical School. The genes encoding the rat M1 wild-type (rM1) and mutant ($rM1^{123}$) muscarinic receptors were stably expressed in CHO cells (Zhu et al., 1991). All cells 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, culture flasks or 10-cm dishes at 37°C in a humidified atmosphere consisting of 5% CO_2 / 95% air.

Pretreatment with agonist

CHO cells expressing hM1-hM4 receptors were incubated with agonists in culture media at 37°C for one minute or one hour, then washed three times with HEPES buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 20 mM HEPES, 58 mM sucrose; pH 7.4 ± 0.02; 340 ± 5 mOsm) to remove any unbound drug. Cells were used immediately or allowed to incubate for 23-24 hours in ligand-free media. Another group of cells was incubated with agonist in culture media for 24 hours before washing three times with HEPES buffer. All pretreatments and radioligand binding assays were performed on cells in monolayer at 37ºC unless otherwise noted.

siRNA treatment

CHO cells expressing hM3 receptors were incubated with 50 nM siRNA each for G_q and G_{11} G-proteins in Opti-Mem for 6-7 hours at 37ºC. Culture medium with no geneticin was added and the cells were allowed to incubate overnight. Subsequent pretreatment with xanomeline was the same as stated under pretreatment with agonist.

Determination of protein content

Protein determinations were performed according to the method of Bradford (Bradford, 1976) following agonist pretreatment. The changes in protein content following agonist treatments were used to correct radioligand binding curves as necessary. Briefly, cells were incubated with carbachol (100 µM – 10 mM), pilocarpine (1 µM – 100 µM), xanomeline (1 µM – 100 µM) or DMSO (0.01 – 1 %) for one hour in HEPES buffer followed by washing, which corresponds to the continuous presence of the agonist in the binding assay. Additional cells were treated with carbachol (100 µM – 10 mM), pilocarpine (1 µM – 100 µM), xanomeline (1 µM – 100 µM) or DMSO (0.01 – 1

%) for one minute or one hour in media followed by washing and used immediately or subsequent to 23-24 hour incubation in control media. Another group of cells was treated with agonists or DMSO for 24 hours prior to washing. In all cases reactions were stopped by incubation with 1 M NaOH for 30 minutes. Samples were either used directly or following dilution in the Bradford assay.

RADIOLIGAND BINDING ASSAYS

Time course

Experiments were conducted to determine the time course of xanomeline wash-resistant binding. CHO hM1-hM4 cells were incubated for various times (< 15 seconds to 30 minutes) with a single concentration of xanomeline (10 μM) and then washed three times with HEPES buffer. In the binding assay, cells were incubated with the muscarinic receptor ligand [^{3}H]N-methylscopolamine ([^{3}H]NMS) (0.2 nM) for one hour at 37ºC. In all instances, non-specific binding was determined using 10 μM atropine during the binding assay. Incubations were terminated by removing the buffer containing free radioligand and then cells with bound radioligand were dissolved in 1M NaOH. Bound radioactivity (disintegrations per minute (dpm)) was quantitated by liquid scintillation spectrometry.

Inhibition of [^{3}H]NMS binding following agonist pretreatments

This protocol was employed to assess agonist potency. In all cases, the IC_{50} (concentration of xanomeline that resulted in the inhibition of 50 percent of radioligand binding) was used as a measure of the potency of xanomeline binding. CHO hM1-hM4 cells were incubated concurrently with 0.2 nM [^{3}H]NMS and increasing concentrations of xanomeline (1 nM – 100 μM), carbachol (10 nM – 10 mM) or pilocarpine (1 nM – 100 μM) for one hour. In order to assess the effects of prolonged agonist treatments, cells

were pretreated with increasing concentrations of xanomeline (1 pM – 100 μM), carbachol (1 pM – 10 mM) or pilocarpine (1 pM – 100 μM) for one minute or one hour followed by washing three times with HEPES buffer and used immediately or after incubation for 24 or 23 hours, respectively, in ligand free media. An additional group of cells was pretreated with agonists for 24 hours prior to washing three times with HEPES buffer. Subsequently, cells were incubated with [^{3}H]NMS (0.2 nM) for one hour. In all instances, pretreatment and radioligand binding were performed in monolayer at 37°C. Non-specific binding was determined using 10 μM atropine. Incubations were terminated as described above. Protein determinations were performed according to the method of Bradford (Bradford, 1976) as described above. Bound radioactivity (dpm) was quantitated by liquid scintillation spectrometry.

Due to the fact that xanomeline was dissolved in dimethyl sulfoxide (DMSO), additional control experiments were performed to ensure that DMSO did not contribute to the observed effects of xanomeline. Radioligand binding and protein content assays were conducted as described above using DMSO concentrations corresponding to those in contact with cells during the various treatment modalities with xanomeline (0.01 – 1%).

Further experiments were conducted at the hM1 and hM3 receptors that were the same as stated above except saturating concentrations of the muscarinic receptor ligands [^{3}H]NMS (2.9 nM) or [^{3}H]QNB (1.4 nM) were used. The concentrations of the [^{3}H]NMS and [^{3}H]QNB were chosen to achieve 93 percent fractional receptor occupancy.

[^{3}H]NMS saturation binding

A saturation binding paradigm was used to determine the total cell-surface receptor density (B_{max}) and radioligand-receptor equilibrium dissociation constant (K_d). CHO hM1-hM4 cells were incubated in monolayer for one hour at 37°C in the absence or presence of xanomeline (10 μM), carbachol (10 μM; hM3 receptor only) or pilocarpine (10 μM; hM3 receptor only) followed by three washes with HEPES buffer and used immediately or after 23 hour incubation in control media. An additional group of cells was treated with xanomeline or carbachol (hM3 receptor only) for 24 hours before washing three times with HEPES buffer. Subsequently, cells were incubated in monolayer with increasing concentrations of [^{3}H]NMS (0.01 – 8.9 nM) for one hour at 37°C. Non-specific binding was determined using 10 μM atropine. Reactions were terminated and bound radioactivity was quantitated as described above. Protein determinations were performed according to the method of Bradford (Bradford, 1976) described above.

<u>FUNCTIONAL ASSAYS</u>

Assay of phosphoinositide (PI) hydrolysis

CHO hM3 or hM1 cells in 24-well plates were loaded with *myo*-[^{3}H]inositol (1 μCi/ml) at 37ºC 24 hours prior to being subjected to several different treatment protocols with agonists and determination of inositol phosphate production.

1) Continuous presence of agonist: Labeled untreated cells were incubated in the presence of increasing concentrations of xanomeline (10 pM – 10 μM), pilocarpine (100 nM – 60 μM), or carbachol (10 nM – 100 μM) in the presence of 10 mM LiCl for one hour at 37ºC.

2) Acute agonist treatment: Cells were pretreated with increasing concentrations of xanomeline (100 pM – 100 μM) for one minute or one hour, at which time cells were washed three times with HEPES buffer. In other cases, cells were treated with a single high concentration of carbachol (10 μM) or pilocarpine (10 μM) for one hour followed by washing. Cells were then incubated for one hour at 37ºC in the presence of 10 mM LiCl but in the absence of further agonist stimulation.

3) Xanomeline treatment followed by stimulation with a second agonist: Cells were pretreated for one minute or one hour with a single concentration of xanomeline (10 μM for hM3; 300 nM for hM1), washed three times with HEPES buffer then used immediately or incubated in control media for 24 or 23 hours, respectively. Additional cells were pretreated with xanomeline (10 μM hM3; 300 nM hM1) for 24 hours, washed and used immediately in the functional assay. Subsequently, cells were incubated with increasing concentrations of carbachol (10 nM – 10 mM), pilocarpine (10 nM – 1 mM; hM3 only) or xanomeline (1 nM - 100 μM; hM3 only) for one hour in the presence of 10 mM LiCl.

4) Agonist treatment followed by stimulation with a second agonist at the hM3 receptor: Cells were pretreated for one hour with a single concentration of carbachol (10 μM) or pilocarpine (10 μM), washed three times with HEPES buffer and used immediately or after 23 hour incubation in control media. Additional cells were pretreated with carbachol (10 μM) or pilocarpine (10 μM) for 24 hours prior to washing three times with HEPES buffer. Subsequently, cells were incubated for one hour with carbachol (10 nM – 10 mM), pilocarpine (10 nM – 1 mM) or xanomeline (1 nM - 100 μM) in the presence of 10 mM LiCl.

5) Time course of agonist stimulation: The time course of xanomeline-induced persistent activation and antagonism of carbachol-elicited PI hydrolysis was established.

Cells were exposed to xanomeline (10 μM for hM3; 300 nM for hM1) for increasing amounts of time (30 minutes – 24 hours) followed by washing and immediate use in the functional assay. Alternatively, cells were treated with xanomeline (10 μM for hM3; 300 nM for hM1) for one hour followed by extensive washing and incubation in control media for increasing lengths of time (1 – 23 hours) as indicated in the Results. In both cases, cells were incubated for one hour in a LiCl-containing buffer in the absence of further agonist stimulation or in the presence of a single high concentration of carbachol (10 μM).

In all cases, reactions were stopped with 0.3 M $HClO_4$ and neutralized with 0.15 M K_2CO_3. The samples were centrifuged (1500 x g; 15 min) and total inositol phosphates in the supernatant 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 and adjusted for ^{14}C recovery.

Control experiments were conducted using CHO hM1 and hM3 cells to insure that the pool of [^{3}H]*myo*-inositol was not depleted following xanomeline pretreatments. To this accord, cells were pretreated with xanomeline as detailed above and then incubated with 2.5 U of exogenous phospholipase C in the presence of 10 mM LiCl. The reactions were stopped and radioactivity quantitated as described above.

Alternatively, CHO cells expressing the hM1 receptor were treated in monolayer but the functional assay was performed in suspension to determine the effect of xanomeline pretreatment on the ability of agonists to further stimulate the production of inositol phosphates. CHO hM1 cells were incubated in monolayer with *myo*-[^{3}H]inositol

(1 µCi/ml) for 24 hours at 37°C prior to being subjected to the following different treatment protocols with agonists and determination of inositol phosphate production.

1) Xanomeline treatment followed by agonist stimulation: Cells were pretreated for one minute or one hour with a single concentration of xanomeline (300 nM), washed three times with HEPES buffer and then used immediately or incubated in control media for 24 or 23 hours, respectively. Additional cells were pretreated with xanomeline (300 nM) for 24 hours prior to washing. Cells were then harvested by trypsinization, centrifuged, and washed three times in HEPES buffer. Subsequently, cells were distributed to assay tubes (500,000 cells /assay tube), and allowed to incubate for 10 minutes at 37°C. Concentration-response curves for the stimulation of inositol phosphate production were determined for carbachol (100 nM – 10 mM), pilocarpine (10 nM – 1 mM) or xanomeline (1 pM – 10 µM) in the presence of 10 mM LiCl.

2) Carbachol treatment followed by agonist stimulation: Cells were pretreated for 24 hours with a single concentration of carbachol (10 µM) prior to washing. Cells were then harvested as described above. Subsequently, cells were incubated for one hour with carbachol (10 nM – 1 mM) or xanomeline (10 pM – 100 µM) in the presence of 10 mM LiCl.

In all cases, the reaction was stopped with chloroform/methanol (2:1) and centrifuged at 450 x g for 15 min. For all experiments, [^{14}C]inositol-1-phosphate was added to each sample as an internal recovery standard. Total inositol phosphates were separated by ion exchange chromatography (AG1-X8 resin). Radioactivity (dpm) in each sample was quantitated by liquid scintillation spectrometry and adjusted for ^{14}C recovery.

VARIATIONS ON RADIOLIGAND BINDING AND FUNCTIONAL ASSAY PARADIGMS

Atropine blockade of receptor activation using saturation binding and functional assays

CHO hM3 cells were grown in 24-well plates and pretreated in monolayer for one hour at 37°C in the absence or in the presence of xanomeline (10 μM) alone or in conjunction with atropine (10 μM) followed by three washes with HEPES buffer and 23 hour incubation in control media. A second group of cells was untreated or treated with xanomeline for one hour, washed extensively and then treated with atropine for 23 hours. In the binding assay, cells were incubated in monolayer with increasing concentrations of [^{3}H]NMS (0.01 - 13.5 nM) for one hour at 37°C. Non-specific binding was determined using 10 μM atropine. Reactions were terminated and radioactivity quantitated as described above. In the functional assay cells were incubated with increasing concentrations of carbachol (10 nM – 10 mM) for one hour in the presence of 10 mM LiCl. The reaction was stopped and radioactivity (dpm) quantitated as described above.

CHO hM1 cells were grown in tissue culture flasks. Pretreatments were performed in monolayer at 37ºC in the absence or in the presence of xanomeline (3 μM saturation; 300 nM function) alone or in conjunction with atropine (10 μM) followed by three washes with HEPES buffer and 23 hour incubation in control media. A second group of cells was untreated or treated with xanomeline for one hour, washed extensively and then treated with atropine for 23 hours. Cells were then harvested by trypsinization, followed by centrifugation (300 x g, 3 min) and re-suspension of the pellet in HEPES buffer (three times). In the suspension binding assay, cells were incubated with increasing concentrations of [^{3}H]NMS (0.02 to 6 nM) for one hour at 37°C using 100,000 cells/assay tube in a total volume of 1 ml. Nonspecific binding was determined

using 10 µM atropine. The reaction was terminated by filtration on Whatman GF/C filters (Whatman Schleicher and Schuell, Keene, NH) using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed three times with 4-ml aliquots of ice-cold saline and dried before radioactivity (dpm) was quantitated using liquid scintillation spectrometry. In the functional assay, 500,000 cells/assay tube were allowed to incubate for 10 minutes at 37ºC prior to the addition of carbachol (100 nM – 1 mM). The reaction was allowed to proceed for one hour in the presence of 10 mM LiCl before being stopped with chloroform/methanol (2:1). The remainder of the procedure was the same as described above for the PI assay in suspension.

Block of receptor activation using a receptor mutant in binding and functional assays

Experiments were conducted to determine the role of receptor function on the long-term effects of xanomeline at the rM1 receptor. CHO rM1 or rM1^{123} mutant cells were exposed to increasing concentrations of carbachol (10 nM – 500 µM) or xanomeline (10 pM – 100 µM) in suspension to determine the functional response. The PI assay was performed as noted previously for cells in suspension. Subsequently, inhibition of [^{3}H]NMS and saturation binding assays were performed. Briefly, in the inhibition of [^{3}H]NMS binding assay, cells were treated concurrently with 0.2 nM [^{3}H]NMS and increasing concentrations of xanomeline (100 pM – 100 µM) for one hour. Additional cells were treated with xanomeline (1 pM – 100 µM) for one hour followed by washing and used immediately or following 23 hour incubation in control media. An additional group of cells was treated with xanomeline for 24 hours prior to washing. The inhibition of [^{3}H]NMS binding assay and radioligand quantification was performed as described previously for binding in suspension. In the [^{3}H]NMS saturation binding

experiments rM1 or $rM1^{123}$ mutant cells were exposed to xanomeline (3 µM) for one hour followed by three washes with HEPES buffer and used immediately or subsequent to 23 hour incubation in the absence of free ligand. An additional group of cells was treated with xanomeline for 24 hours prior to washing. The radioligand binding assay and quantification were performed as previously described for suspension binding. Control experiments utilizing DMSO in [^{3}H]NMS binding and protein assays were conducted as described above to determine the effects of agonist pretreatments on rM1 or $rM1^{123}$ mutant receptors.

Use of siRNA blockade of receptor function in saturation binding and functional assays

Experiments were conducted to determine the role of receptor function on the long-term effects of xanomeline at the M3 receptor. siRNA transfections were performed as noted above. The transfection protocol was unsuccessful in cells seeded in 24-well plates. Therefore, transfections were performed in 10-cm dishes. To confirm a decrease in inositol phosphates production, non-transfected and transfected cells were removed from the 10-cm dishes by trypsinization and centrifuged (300 x g, 2 min) and re-suspended three times in HEPES buffer. Subsequently, in the functional assay in suspension, cells were incubated with carbachol (1 µM and 100 µM) or xanomeline (100 nM and 10 µM) for one hour at 37ºC. The reaction was stopped and the accumulation of inositol phosphates was determined as previously stated for suspension.

Western blot analysis was performed to confirm a decrease in $G_{q/11}$ G protein expression following siRNA pretreatment. Briefly, cells were washed and centrifuged. The pellet was reconstituted in 250 µl of RIPA lysis buffer and placed on ice for 20 minutes. Cells were passed through a 23-guage needle to further homogenize the

sample, then incubated on ice for 20 minutes and spun at 14,000 x g for 20 minutes at 4°C. The supernatant was collected and the amount of protein in the sample was determined using the method of Bradford (Bradford, 1976). Fifty μg of protein from each sample was loaded onto a 12.5% SDS polyacrylamide gel. The proteins were separated by electrophoresis then transferred onto a nitrocellulose membrane, which was blocked overnight at 4°C with a dry milk solution. The $G_{q/11}$ G proteins were detected with a rabbit anti-$G_{q/11}$ antibody. Horseradish peroxidase-labeled anti-rabbit secondary antibody was used. The membrane was then treated with chemiluminescence reagents and exposed on x-ray film. The approximately 45 kD band was quantified using a Bio-Rad densitometer. The optical density of each sample was measured followed by subtraction of background.

Subsequently, in the [^{3}H]NMS saturation binding assay, non-transfected or siRNA transfected cells were incubated for one hour at 37°C in the absence or presence of xanomeline (10 μM) followed by washing and immediate use or further incubation for 23 hours in control medium. Additional cells were treated with xanomeline for 24 hours prior to washing three times with HEPES buffer. Cells were then removed from the 10 cm dishes by trypsinization, centrifuged (300 x g, 2 min) and re-suspended in HEPES buffer (three times). Subsequently, cells were incubated with increasing concentrations of [^{3}H]NMS (0.04 – 8.5 nM) in suspension for one hour at 37°C. The remaining details are the same as previously described for saturation binding in suspension.

[^{3}H]NMS saturation binding following propyl benzyl choline mustard (PrBCM) treatment

Experiments were conducted to determine the effect of decreasing the level of cell-surface receptor density on saturation binding parameters using an irreversible

receptor alkylating agent. The levels of cell-surface receptor density at the M4 receptor were decreased to the levels observed at the M2 receptor to determine if a lower level of receptor density had an influence on the long-term effects of xanomeline binding. To determine the concentration of propylbenzyl choline mustard (PrBCM) that resulted in an approximately 50 percent decrease in receptor expression CHO cells expressing the hM4 receptor were treated with increasing concentrations of 0.1 – 10 nM cyclized PrBCM (Hu & El-Fakahany, 1990) for 15 minutes, followed by washing three times with HEPES buffer. Subsequently, cells were exposed
to 6.5 nM [^{3}H]NMS for one hour at 37ºC. The reaction was stopped and radioligand binding was determined as described above for binding assays in monolayer. Subsequent saturation binding experiments were conducted using 2 nM PrBCM. In this case cells were exposed to 2 nM PrBCM for 15 minutes followed by three washes with HEPES buffer. Cells were then pretreated with xanomeline (10 μM) for one hour followed by washing and waiting 23 hours or treated with xanomeline for 24 hours prior to washing. Subsequently, untreated or xanomeline pretreated cells were exposed to increasing concentrations of [^{3}H]NMS (0.03 – 7 nM) for one hour at 37ºC. The rest of the protocol for the termination of the reaction and radioligand quantitation is as described above for saturation binding assays in monolayer.

[^{3}H]NMS saturation binding following pretreatment with pertussis toxin

Experiments were conducted to determine the effect of blocking the $G_{i/o}$ G proteins on saturation binding parameters at the hM4 receptor. Cells were treated with pertussis toxin (100 ng/ml) in media for 24 hours. Subsequently, cells were pretreated with xanomeline (10 μM) for one hour followed by washing three times with HEPES buffer and further incubation for 23 hours in ligand free media containing pertussis toxin.

Additional cells were treated concurrently with xanomeline and pertussis toxin for 24 hours prior to washing. Cells were then treated with increasing concentrations of $[^3H]$NMS (0.02 – 7.5 nM) for one hour at 37ºC. The reaction was stopped and radioligand binding determined as described above for saturation binding on cells in monolayer.

DATA ANALYSIS

All analyses were conducted using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Inhibition of $[^3H]$NMS binding isotherms were analyzed via nonlinear 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 curves were analyzed via nonlinear regression to derive individual estimates of B_{max} (total cell-surface receptor density) and K_d (radioligand-receptor equilibrium dissociation constant). PI hydrolysis isotherms were analyzed via non-linear regression. Data shown are the means ± standard error of the mean. Comparisons between mean values were performed by paired *t* tests, unpaired *t* tests or one way analysis of variance (ANOVA), as appropriate. A probability (p) value < 0.05 was taken to indicate statistical significance.

CHAPTER 3: Determination of the acute and long-term effects of xanomeline binding at M1-M4 muscarinic receptors

Introduction

Muscarinic acetylcholine receptors (mAChR) are part of the G protein-coupled receptor superfamily. There are five subtypes of muscarinic receptors (M1-M5) (Caulfield & Birdsall, 1998). These receptors are expressed widely throughout the brain and periphery (Caulfield & Birdsall, 1998). Each receptor has a different primary physiological function with the M1 receptor involved in learning and memory (Eglen, 2005). M2 and M4 receptors are mainly located presynaptically and mediate autoinhibition of acetylcholine release in the hippocampus and cerebral cortex, respectively (Wess et al., 2003). M3 receptors are involved in salivation, food intake, and gastrointestinal tract motility and secretion (Eglen, 2005). M5 receptors facilitate dopamine release in the striatum (Wess et al., 2003). There is a high degree of homology between the orthosteric (primary) binding region across subtypes (Eglen, 2005), which makes it difficult to develop an agonist that binds selectively to a particular subtype (Wess, 2004). However, recent research suggests that there are secondary binding domains (allosteric binding sites) on muscarinic receptors that are less conserved across subtypes, which may lead to the development of agonists that are able to act in a more selective manner (Eglen, 2005). Prolonged exposure of receptors to an agonist can lead to changes in the binding properties of the receptor. These changes in binding properties can result from receptor internalization, down-regulation or uncoupling from the G protein (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990).

Xanomeline is a potent agonist that is able to bind to all subtypes of muscarinic receptors with similar potency (Shannon et al., 1994). However, it is thought to have functional selectivity for the M1 and M4 receptors (Bymaster et al., 1997; Shannon et al., 1994). However, it should be noted that the previous research was conducted using different receptor expression systems. Additionally, xanomeline binding at the M1

receptor has been shown to take place both at the orthosteric (primary) binding site in a reversible manner and at an allosteric (secondary) site in a wash-resistant manner (Christopoulos et al., 1998). However, it is unclear if binding of xanomeline occurs through the same mechanisms at other receptor subtypes.

We were interested in determining the short- and long-term effects of xanomeline binding across receptor subtypes. It is important to compare the effects of xanomeline binding in the same expression systems to avoid complications caused by factors not related to the type of muscarinic receptor expressed. Thus, experiments were conducted in Chinese hamster ovary (CHO) cells expressing M1-M4 muscarinic receptors to test the hypotheses that 1) xanomeline binds to all receptor subtypes in both a reversible and wash-resistant manner, 2) xanomeline wash-resistant binding results in long-term effects similar to those seen during prolonged treatment with agonists, such as internalization and down-regulation and 3) there is subtype selectivity in the long-term effects of wash-resistant xanomeline binding.

Results

Time course of development of wash-resistant xanomeline binding at M1-M4 receptor subtypes

The time course of xanomeline binding in a wash-resistant manner was determined at each of the muscarinic receptor subtypes expressed in CHO cells by measuring the decrease in [^{3}H]NMS binding over time following xanomeline exposure and extensive washing. [^{3}H]NMS binding in the absence of xanomeline was used as a control. In all cases, there was an immediate decrease in [^{3}H]NMS binding when cells were briefly treated (< 15 seconds) with 10 μM xanomeline (Fig. 1). However, the decrease in [^{3}H]NMS binding following xanomeline treatment for <15 seconds was

significantly ($p < 0.05$) greater for the M1 (48%) and M3 (41%) receptor subtypes, than the M2 (24%) or M4 (30%) subtypes, suggesting that xanomeline wash-resistant binding occurs more rapidly at the former subtypes. Subsequently, there was a slower phase of inhibition of [^{3}H]NMS binding that reached a plateau by approximately 10 minutes for all subtypes. Once again, the maximal decrease in [^{3}H]NMS binding was significantly ($p < 0.05$) greater at the M1 (81%) and M3 (83%) receptors, compared to the M2 (66%) and M4 (71%) receptors (Fig. 1). The results at the M1 receptor are in agreement with what Jakubik et al. (2002, 2006) observed. However, there is a discrepancy regarding the time course of xanomeline binding at the M2 receptor. Specifically, Jakubik et al. (2006) observed no decrease in [^{3}H]NMS binding following treatment with xanomeline for <15 seconds and a more rapid decrease in the subsequent phase. These differences could be the result of variations in the experimental protocols employed. In the current protocol, the binding assay was performed on intact cells in monolayer whereas the protocol used by Jakubik et al. (2006) for the binding assay was one in which treated cells had been homogenized to create membrane preparations for the binding assay. Additionally, they used a higher concentration of [^{3}H]NMS (1 nM), which could influence the ability of xanomeline to compete with and inhibit radioligand binding.

Differential effects of acute xanomeline treatment on inhibition of [^{3}H]NMS binding at M1-M4 receptor subtypes

Previous research from our laboratory and others has demonstrated that xanomeline binding occurs in a wash-resistant manner at the M1 receptor (Christopoulos et al., 1998; Grant & El-Fakahany, 2005; Jakubik et al., 2002). The current set of experiments were undertaken to compare the short-term effects of xanomeline binding across the M1-M4 receptor subtypes. The presence of xanomeline in the binding assay

medium caused a concentration-dependent and complete inhibition of [^{3}H]NMS binding at all receptor subtypes (Fig. 2). The rank order of xanomeline potencies was M3 ≈ M4 > M2 > M1. When cells were treated with xanomeline for one minute or one hour followed by washing and immediate use there was a significant ($p < 0.05$) decrease in xanomeline potency for all subtypes compared to the experimental protocol described above (Fig. 2; Table 1). Pretreatments for one minute, but not for one hour, resulted in incomplete inhibition of [^{3}H]NMS binding at all four receptor subtypes (Table 1). For the brief one minute xanomeline pretreatments, the rank order of potencies of xanomeline was M1 > M2 > M3 ≈ M4. However, there was no difference in the potencies when the treatment time with xanomeline was increased to one hour.

Since xanomeline was dissolved in dimethylsulfoxide (DMSO), control experiments were conducted to ascertain if the vehicle contributed to any of the observed inhibition of [^{3}H]NMS binding. The concentrations of DMSO used corresponded to those employed in the preparation of the various xanomeline concentrations. DMSO, up to 1%, had no significant effect on [^{3}H]NMS binding under any of the experimental paradigms described above (Fig. 3).

We initially noted that there were visual decreases in cell density when cells were observed using a microscope following xanomeline pretreatments at the M1 receptor. To correct for the effects of the decrease in cell density on [^{3}H]NMS binding we assayed protein content using the method of Bradford (Bradford, 1976) and corrected the binding curves for the changes in protein content if necessary. When cells were pretreated continuously with xanomeline in HEPES for one hour, mimicking the control treatment, there was no change in protein content at any of the receptor subtypes. Additionally, if cells were treated for one minute or one hour with xanomeline in culture media followed by washing there was no change in protein expression at any of the receptor subtypes

(Fig. 4). Thus, correction of the binding curves was not necessary following acute xanomeline treatments. When similar experiments were conducted using DMSO concentrations corresponding to those used in the preparation of xanomeline there was also no change in protein content for any of the treatment groups (Fig. 5).

Effects of acute carbachol treatments on inhibition of [^{3}H]NMS binding at M1-M4 receptor subtypes

Carbachol was employed in place of xanomeline in the pretreatment paradigm to determine if the observed effects of xanomeline pretreatments on [^{3}H]NMS binding were unique to xanomeline or are a general property of muscarinic agonists. Carbachol was used because it is a full agonist at all of the receptor subtypes studied (Wang & El-Fakahany, 1993). There was a concentration-dependent and complete inhibition of [^{3}H]NMS binding when carbachol was present concurrently with 0.2 nM [^{3}H]NMS for one hour, similar to that seen with xanomeline albeit with a lower potency for all subtypes (Fig. 6; Table 1). In contrast, when cells were exposed to carbachol for one hour followed by washing and immediate use there was no significant decrease in [^{3}H]NMS binding for any of the receptor subtypes, with the exception of cells expressing the M4 receptor, which had a concentration-dependent decrease in [^{3}H]NMS binding (Fig. 6 d). Results are summarized in Table 1. These results are in stark contrast to those of acute wash-resistant xanomeline interaction with M1-M4 receptors (Fig. 2).

Control experiments were conducted to ascertain if pretreatment with carbachol had an effect on protein expression at any of the receptor subtypes. Cells were pretreated as described in the previous paradigm. Similar to the results presented above, acute pretreatment with carbachol had no effect on protein content for any of the subtypes (Fig. 7).

Effects of acute pilocarpine treatment on inhibition of [^{3}H]NMS binding at the M3 receptor subtype

Additional experiments were conducted at the M3 receptor to determine the effects of increasing concentrations of a partial agonist, pilocarpine, on the inhibition of [^{3}H]NMS binding, since xanomeline is a partial agonist at the M3 receptor, whereas it is a full agonist at the M1 receptor (Chapter 4 Figs. 1 & 2). The results with pilocarpine were similar to those of carbachol at the M3 receptor. There was a concentration-dependent and complete inhibition of [^{3}H]NMS binding when pilocarpine was present concurrently with [^{3}H]NMS (Fig. 8; Table 1). However, when cells were treated with pilocarpine for one hour followed by washing there was no decrease in [^{3}H]NMS binding (Fig. 8). Additionally, pretreatment with pilocarpine using the same paradigms described previously did not have an effect on protein expression for either of the treatment groups (Fig. 9).

The effect of acute xanomeline treatment on [^{3}H]NMS saturation binding at M1-M4 receptor subtypes

In subsequent experiments, cells expressing M1-M4 receptors were incubated with increasing concentrations of [^{3}H]NMS following treatment with 10 μM xanomeline to determine the consequence of xanomeline binding on cell-surface receptor density and radioligand-receptor affinity. In all cases, when cells were treated with 10 μM xanomeline for one hour followed by washing and immediate use there was no significant change in the cell-surface receptor density; however, there was a significant ($p < 0.05$) decrease in radioligand-receptor affinity compared to control (Fig. 10; Table 2). Noteworthy, the decrease in radioligand affinity was greatest at the M1 receptor, followed closely by the M3 receptor and then the M4 and M2 receptors (Table 2).

Differential effects of long-term xanomeline treatment on inhibition of [^{3}H]NMS binding at M1-M4 receptor subtypes

Prolonged continuous exposure to agonists has been demonstrated to lead to receptor internalization, down-regulation or uncoupling of the receptor from the G protein (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990). These effects can result in changes in the binding properties of the receptor. In subsequent experiments, we were interested in determining the long-term effects of brief and prolonged exposure to xanomeline on the binding properties across muscarinic receptor subtypes. In contrast to brief treatments followed by washing discussed above, when cells were treated with xanomeline for one minute followed by washing and incubation for 24 hours in control media a biphasic binding curve was observed at the M1 and M3 receptor subtypes, but not at the M2 or M4 receptor subtypes (Fig. 11). Complete inhibition of [^{3}H]NMS was still observed at all receptor subtypes (Table 1). When the incubation time with xanomeline was increased to one hour prior to washing and waiting 23 hours, a biphasic binding curve was also observed at all but the M2 receptor (Fig. 11). Interestingly, there was complete inhibition of [^{3}H]NMS binding at all but the M3 receptor under these conditions (Table 1). Similarly, when cells were incubated with xanomeline for 24 hours prior to washing a biphasic binding curve was observed at all but the M2 receptor. The high-potency site accounted for approximately 44, 41, and 29 percent of the total receptor population for the M1, M3 and M4 receptor subtypes, respectively. Interestingly, the high-potency binding site was of markedly higher potency for 24 hour xanomeline pretreatment than was observed with the acute (one minute or one hour) xanomeline pretreatments ($p < 0.05$) (Table 1). Under these conditions, inhibition of [^{3}H]NMS binding was complete at all subtypes (Table 1).

As described above for acute xanomeline treatments, control experiments using DMSO in the pretreatment paradigms were conducted to insure that the vehicle did not contribute to the observed inhibition of $[^3H]$NMS binding. The presence of the various concentrations of DMSO used in the preparation of xanomeline had no effect on $[^3H]$NMS binding for any of the previously described treatment paradigms (Fig. 12).

We initially noted that there were visual changes in cell density following xanomeline pretreatments at the M1 receptor. A decrease in protein content would necessitate correction of the binding curves for protein content. The method of Bradford (Bradford, 1976) was used to assess protein content and corrections to the binding curves were made as necessary. As stated above, when cells were pretreated continuously with xanomeline in HEPES buffer for one hour (control) there was no change in protein content at any of the receptor subtypes (Fig. 4). However, when cells expressing the M1 receptor were treated with xanomeline for one minute or one hour followed by washing and overnight incubation in ligand free media there was a concentration dependent decrease in protein content (Fig. 13 a). Similarly, 24 hour-treatment followed by washing also resulted in a decrease in protein content at the M1 receptor. Thus, the binding curves at the M1 receptor were corrected as a result of xanomeline pretreatment. In contrast, when the same pretreatments were performed at the M2-M4 receptors there was no change in protein content for any of the treatment paradigms (Fig. 13 b-d). When similar experiments were conducted utilizing the same concentrations of DMSO used in the preparation of xanomeline there was no change in protein content for any of the pretreatment paradigms described previously at the various receptor subtypes (Fig. 14).

Effects of long-term carbachol treatments on inhibition of [^{3}H]NMS binding at the M1-M4 receptor subtypes

To ascertain whether the long-term effects of xanomeline binding were specific to xanomeline or are a general property of muscarinic agonist binding, experiments were undertaken using the full agonist carbachol. When cells were exposed to increasing concentrations of carbachol for one hour followed by washing and further incubation for 23 hours in control media there was no decrease in [^{3}H]NMS binding for any of the receptor subtypes (Fig. 15). This is in stark contrast to the delayed appearance of biphasic binding curves at all but the M2 receptor subtype with xanomeline treatment (Fig. 11). However, when cells were exposed to carbachol for 24 hours prior to washing there was a concentration-dependent decrease in [^{3}H]NMS binding at all of the subtypes (Fig. 15). The maximal decrease in binding was significantly ($p < 0.05$) greater for the M1 and M2 receptors in comparison to the M3 and M4 receptors. The results are summarized in Table 1. Again, this was in stark contrast to the effects of 24 hour-treatment with xanomeline prior to washing that resulted in a biphasic binding curve at all except the M2 receptor and complete inhibition of [^{3}H]NMS binding at all subtypes (Fig. 11). These data indicate that xanomeline binding results in long-term effects that are distinct from the conventional reversible agonist carbachol. To determine if there were changes in protein content following any of the long-term exposure to carbachol paradigms protein assays using the method of Bradford (Bradford, 1976) were conducted. There was a decrease in protein content at the M1 receptor following 24 hour carbachol treatment, thus necessitating corrections be made to the binding curve (Fig. 16 a). However, there was no change in protein for any of the other previously described carbachol pretreatments across receptor subtypes (Fig. 16).

Effects of long-term pilocarpine on inhibition of [^{3}H]NMS binding at the M3 receptor subtype

Additional experiments were conduced at the M3 receptor using pilocarpine in the inhibition of [^{3}H]NMS binding assay instead of carbachol to further determine if the long-term effects of exposure to a conventional agonist are different from xanomeline. As noted previously, pilocarpine is a partial agonist at the M3 receptor. When cells were exposed to increasing concentrations of pilocarpine for one hour followed by washing and waiting 23 hours there was no decrease in [^{3}H]NMS binding (Fig. 17). However, when cells were pretreated with pilocarpine for 24 hours prior to washing there was a concentration-dependent decrease in [^{3}H]NMS binding similar to that observed with carbachol, however the potency was lower for pilocarpine compared to carbachol (Fig. 17). Results are summarized in Table 1. Taken together, these data demonstrate that the long-term effects of xanomeline binding are distinct from those of conventional muscarinic agonists. When experiments were conducted to determine protein content following pilocarpine treatment paradigms as discussed above, no differences were seen between any of the pretreatment groups (Fig. 18).

The effects of long-term xanomeline treatment on [^{3}H]NMS saturation binding at M1-M4 receptor subtypes

Experiments were conducted to determine the long-term effects of 10 μM xanomeline exposure on cell-surface receptor density and radioligand-receptor affinity. When cells were treated with xanomeline for one hour followed by washing and incubation for 23 hours in the absence of free ligand there was no change in radioligand-receptor affinity at any of the subtypes, but a significant ($p < 0.05$) decrease in cell-surface receptor density at all except the M2 receptor (Fig. 19). When cells were treated

with xanomeline for 24 hours prior to washing there was a significant ($p < 0.05$) decrease in radioligand-receptor affinity across all four subtypes and a significant ($p < 0.05$) decrease in cell-surface receptor density at all except the M2 subtype (Fig. 19). Interestingly, the decrease in cell-surface receptor density was more pronounced at the M1 receptor compared to the M3 or M4 receptors. Results are summarized in Table 2.

The effects of long-term conventional agonist treatment on [^{3}H]NMS saturation binding parameters at the M3 receptor

Additional experiments were conducted to determine if the effects of prolonged exposure of cells to the conventional agonists carbachol or pilocarpine are distinct from those of xanomeline. Previous research has shown that prolonged exposure of cells expressing muscarinic receptors to carbachol leads to a decrease in cell-surface receptor density with no change in radioligand affinity (Cioffi & El-Fakahany, 1989; Hu et al., 1991; May et al., 2005; van de Westerlo et al., 1995; Wang et al., 1990; Yang et al., 1993). To confirm these results in our experimental paradigm, we determined the effects of long-term exposure of M3 receptors to carbachol. CHO cells expressing the M3 receptor were subjected to the various pretreatments and washing with 10 μM carbachol, then exposed to increasing concentrations of [^{3}H]NMS in order to determine changes in cell-surface receptor density and radioligand affinity . When cells were treated with carbachol for one hour followed by washing and incubation in control media for 23 hours, there was no change in the maximal binding or radioligand affinity compared to control cells (Fig. 20 a). In contrast, pretreatment with xanomeline under the same conditions resulted in a decrease in cell-surface receptor density (Fig. 19). In agreement with previous literature (Hu et al., 1991; May et al., 2005; van de Westerlo et al., 1995; Yang et al., 1993), pretreatment with carbachol for 24 hours prior to washing

resulted in a decrease in maximal binding. However, there was a significant ($p < 0.05$) increase in radioligand affinity (Fig. 20 a; Table 2). This is in stark contrast to the marked decrease in radioligand affinity observed following pretreatment with xanomeline for 24 hours (Fig. 19).

Previous research regarding the effects of prolonged exposure to pilocarpine has produced mixed findings. Shifrin & Klein (1980) found that prolonged exposure to pilocarpine resulted in a decrease in receptor density. In contrast, another report suggested that pilocarpine has little effect on the actual number of receptors, as the apparent down-regulation was due to pilocarpine being released when cells were lysed prior to the binding assay (Milligan & Strange, 1983). In our paradigm, when cells were exposed to 10 μM pilocarpine for one hour followed by washing and waiting 23 hours there was a slight, but significant ($p < 0.05$), decrease in cell surface receptor density without a change in receptor affinity (Fig. 20 b). Additionally in our paradigm, 24 hour-exposure of cells to pilocarpine prior to washing resulted in a significant ($p < 0.05$) decrease in receptor density along with a significant ($p < 0.05$) increase in radioligand affinity (Fig. 20 b). Results are summarized in Table 2. These results further demonstrate a unique binding profile following prolonged exposure to xanomeline compared to conventional agonists.

Discussion

The high degree of sequence homology of the orthosteric binding site of various subtypes of muscarinic receptors has made it difficult to develop an agonist that binds with selectivity at a particular subtype (Wess, 2004). Xanomeline is thought to be an M1/M4 functionally selective agonist, although it can bind at all subtypes (Bymaster et al., 1997; Shannon et al., 1994). Xanomeline has been shown to bind in a unique

manner, with both reversible and wash-resistant binding at the M1 receptor (Christopoulos et al., 1998). Thus, experiments were undertaken to determine the short- and long-term effects of xanomeline binding at the M1-M4 muscarinic receptor subtypes expressed in CHO cells.

Our results indicate that wash-resistant binding occurs rapidly (within 15 seconds) at all four receptor subtypes studied (Fig. 1). However, the immediate decrease in [^{3}H]NMS binding was greater at the M1 and M3 subtypes compared to the M2 and M4 subtypes, as was the maximal inhibition of [^{3}H]NMS binding. It is possible that the differences in the speed of binding are a result of differences in the structure of the allosteric site on receptor subtypes. Alternatively, it might be due to differences in receptor conformation induced by receptor subtype coupling to different G proteins and the subsequent activation of different signaling pathways. Both the M1 and M3 receptors couple to $G_{q/11}$ G proteins (Volpicelli & Levey, 2004) whereas the M2 and M4 receptors couple to $G_{i/o}$ G proteins (Eglen, 2005).

In agreement with previous research (Bymaster et al., 1997; Shannon et al., 1994) our results demonstrate that xanomeline is able to bind to all muscarinic receptor subtypes. This can be seen as a concentration-dependent decrease in [^{3}H]NMS binding at all four receptor subtypes when xanomeline is present concurrently with the radioligand in the binding assay (Fig 2). In addition, when cells were incubated with xanomeline for one minute or one hour prior to washing and immediately used in the binding assay there was a concentration-dependent decrease in [^{3}H]NMS binding, demonstrating the ability of xanomeline to bind in a persistent manner at all subtypes. This is in agreement with previously published data that wash-resistant binding occurs at the M1 (Christopoulos & El-Fakahany, 1997), and M2 (Jakubik et al., 2002; Jakubik et al., 2006) receptors. Interestingly, the rank order of potencies at various receptor

subtypes is different between the various xanomeline treatments. When cells are treated concurrently with xanomeline and [^{3}H]NMS the rank order is M3 ≈ M4 > M2 > M1. This protocol detects both xanomeline reversible and wash-resistant binding. The reverse rank order of potency (M1 > M2 > M3 ≈ M4) was observed when cells are pretreated with xanomeline for one minute followed by washing and immediate use. This might be explained, at least in part, by different kinetics of the rapid phase of xanomeline binding at the different subtypes, especially since differences in potency among subtypes disappear when cells are treated with xanomeline for one hour followed by washing and immediate determination of radioligand binding.

When parallel experiments were undertaken utilizing carbachol in place of xanomeline in the various pretreatment paradigms major differences were observed in the binding profiles of the two agonists (Fig 6). There was a concentration-dependent decrease in [^{3}H]NMS binding with carbachol when concurrently present with the radioligand at all subtypes, similar to that seen with xanomeline; however, the potency was markedly decreased. Carbachol pretreatment for one hour followed by washing and immediate use did not results in a decrease in [^{3}H]NMS binding for any of the subtypes, except when cells expressing the M4 receptor were treated for one hour followed by washing and immediate use. This was in stark contrast to the monophasic binding curves observed with acute xanomeline treatments. The decrease in [^{3}H]NMS binding observed at the M4 receptor following acute carbachol treatment could be due to rapid internalization of the receptor (Feigenbaum & El-Fakahany, 1984; Holroyd et al., 1999). Additionally, pretreatment with pilocarpine at the M3 receptor resulted in a binding profile similar to that observed with carbachol (Fig. 8). Together these results demonstrate that xanomeline interacts with muscarinic receptors in a manner that is distinct from conventional agonists.

Xanomeline was shown to bind in a wash-resistant manner at all four receptor subtypes. Thus, additional experiments were undertaken to determine if the acute wash-resistant binding of xanomeline leads to subtype-dependent changes in receptor density or radioligand affinity. Interestingly, there was no change in receptor density following acute xanomeline treatment compared to control at any of the receptor subtypes (Fig. 10). Similarly, all receptor subtypes demonstrated a decrease in radioligand affinity following acute treatment with xanomeline, albeit to slightly different degrees. Taken together, these data demonstrate that the effects of acute xanomeline treatment are similar across muscarinic receptor subtypes.

Regulation of receptors is time dependent, with receptor internalization taking place at shorter durations of exposure to agonists, and down-regulation predominating at longer incubation times (Maloteaux & Hermans, 1994). In addition, allosteric modulators can modify the conformation of the orthosteric site resulting in changes in the binding properties of additional agonists or antagonists (Christopoulos, 2002). Thus, the effects of prolonged treatment with xanomeline were investigated across the four receptor subtypes. Treatment of cells with xanomeline for one minute followed by washing and incubation in control media for 24 hours results in a monophasic binding curve for the M2 and M4 receptors, but a biphasic binding curve at the M1 and M3 receptors (Fig. 11). However, increasing the pretreatment time to one hour prior to washing and waiting results in biphasic binding curves at all but the M2 receptor (Fig. 11). The observed differences in the long-term effects of exposure of M4 receptors to xanomeline for one minute or one hour suggest a time-dependent interaction of xanomeline with different receptor conformations. Long-term treatment with agonists has been shown to result in internalization/down-regulation of receptors (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990). Thus, the development of the apparent high-potency site at the M1, M3 and M4

receptors could be a reflection of internalization/down-regulation of the receptor that results in a decrease in radioligand binding to the receptor. In support of this notion, the effects of one hour pretreatment with xanomeline followed by washing and waiting were mimicked by treatment with xanomeline for 24 hours. However, the latter condition resulted in higher potencies at both the high and low potency sites.

Parallel experiments utilizing carbachol in place of xanomeline in the binding paradigms described above further demonstrated that xanomeline interacts with the muscarinic receptors in a unique manner. Carbachol pretreatment for one hour followed by washing and further incubation for 23 hours in control media did not result in a decrease in [^{3}H]NMS binding for any of the subtypes. When cells were treated with carbachol for 24 hours followed by washing there was a concentration-dependent decrease in [^{3}H]NMS binding at all of the subtypes, however, the resultant decrease was best-fit by a monophasic binding curve (Fig. 15). The results of either of these treatments were in stark contrast to the biphasic binding curve observed with xanomeline pretreatments under the same conditions. Interestingly, exposure of cells expressing the M3 receptor to pilocarpine using the various pretreatment paradigms results in a binding profile similar to that seen with carbachol, albeit with different potencies (Fig. 17). Taken together these results further demonstrate that long-term exposure to xanomeline results in a ligand-receptor interaction that is distinct from that induced by conventional muscarinic receptor agonists. Additionally, receptor modulation of the M2 receptor by xanomeline is distinctly different from the other receptor subtypes. The results following the exposure of M2 receptors to carbachol demonstrate that the lack of similar long-term effects of xanomeline at the M2 receptor compared to the other subtypes is not due to insensitivity of this receptor to agonist-induced regulation, since the effects of carbachol are similar to those seen at other subtypes. We propose that

the lack of long-term effects of xanomeline at the M2 receptor may be due to absence or a very minimal functional response to xanomeline. It is also important to consider that since xanomeline is able to bind, but stimulates a minimal if any functional response, it may have antagonistic properties at the M2 receptor.

To further support the idea that the effects of xanomeline may be a result of allosteric receptor modulation, the ability of xanomeline to inhibit [^{3}H]NMS binding was studied. Incomplete inhibition of radioligand binding is a hallmark of allosteric receptor modulation (Lee & El-Fakahany, 1991). When cells were treated with xanomeline for one minute or one hour followed by washing and waiting there was incomplete inhibition of [^{3}H]NMS binding at all subtypes, except for one hour treatment at the M3 receptor (Fig. 11). Thus, it is possible that the long-term effects of xanomeline binding are due to changes in cooperativity between the orthosteric and allosteric binding sites. However, it is also possible that the lack of complete inhibition of [^{3}H]NMS binding is a result of the inability to test higher concentrations of xanomeline. Interestingly, when cells were treated with xanomeline for 24 hours prior to washing there was once again complete inhibition of [^{3}H]NMS binding suggesting receptor modulation through internalization/down-regulation (Table 1).

Further experiments were undertaken to quantitate the effects of xanomeline binding on cell-surface receptor density and radioligand affinity (Fig. 19). When cells expressing M1, M3 or M4 receptors were exposed to xanomeline for one hour followed by washing and 23 hour incubation in ligand-free media or treated with xanomeline for 24 hours prior to washing there was a decrease in cell-surface receptor density. However, the decrease was more distinctive at the M1 receptor compared to the other subtypes. These results are in agreement with the results of experiments of inhibition of [^{3}H]NMS binding in which the M1 receptor had a more distinctive high-potency binding

site compared to the M3 or M4 receptors (Fig. 11). Prolonged exposure to agonists has been shown to lead to receptor internalization and/or down-regulation (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990). This provides further support to the notion that the decrease in cell-surface receptor density following long-term xanomeline exposure could be a result of internalization or down-regulation of the receptor. In contrast, neither xanomeline pretreatment condition had an effect on cell-surface receptor density in cells expressing the M2 receptor. This further demonstrates that the long-term regulation of the M2 receptor by xanomeline is distinct from the other receptor subtypes and suggests the possibility that xanomeline is unable to activate the M2 receptor. In addition, it should be noted that the cell-surface receptor density levels in our experimental system are similar for the M1, M3 and M4 receptors, but are considerably lower at the M2 receptor (Table 2). The decreased cell surface receptor density at the M2 receptor is not likely to lead to the large differences in the effect of long-term xanomeline exposure compared to the other receptor subtypes. These differences are more likely to be the result of varied efficacy of xanomeline at various receptor subtypes. Previous work by Shannon et al. (1994) found that xanomeline had little efficacy, if any, in heart tissue that predominantly has M2 receptors. Additionally, recent results quantifying ERK phosphorylation showed that xanomeline only elicited 40% of the functional response at the M2 receptor compared to the M4 receptor in cells that had similar receptor expression (Arthur Christopoulos, personal communication). In contrast to effects on receptor expression, radioligand affinity was decreased following 24 hour xanomeline treatment prior to washing at all four subtypes. This suggests that the change in radioligand affinity is dependent solely on binding of xanomeline to the receptor.

Parallel experiments were conducted at the M3 receptor using carbachol or pilocarpine in place of xanomeline in the pretreatment paradigms. Previous research

has demonstrated that exposure of cells to carbachol for 24 hours results in a decrease in cell-surface receptor density (Hu et al., 1991; May et al., 2005; van de Westerlo et al., 1995; Yang et al., 1993). In either case, pretreatment for one hour followed by washing and waiting resulted in no change in radioligand affinity and only a slight decrease in receptor density following pilocarpine treatment. Twenty-four hour treatment with either carbachol or pilocarpine resulted in a decrease in cell-surface receptor density; however, this decrease was less marked than the decrease observed with the xanomeline treatment following the same experimental protocol. Additionally, 24-hour treatment with either agonist resulted in an increase in radioligand affinity, which is in stark contrast to the decrease in radioligand affinity observed with xanomeline treatment. Together, these results further demonstrate that xanomeline interacts in a unique manner with muscarinic receptors.

In summary, we have shown that although xanomeline can bind in both a reversible and wash-resistant manner with near equal potency across the M1-M4 receptor subtypes, the long-term effects of xanomeline binding are distinctively different at the M2 receptor compared to the other receptor subtypes. The differences in the long-term effects of xanomeline are most likely due to a difference in xanomeline efficacy between subtypes. Additionally, the effects of xanomeline treatment are distinctively different from the effects of treatment with conventional muscarinic receptors employing the same paradigms. Studies described in subsequent chapters are aimed at determining the effects of xanomeline on the functional properties of the M1 and M3 receptors and the mechanisms of receptor regulation by xanomeline.

Figure 1

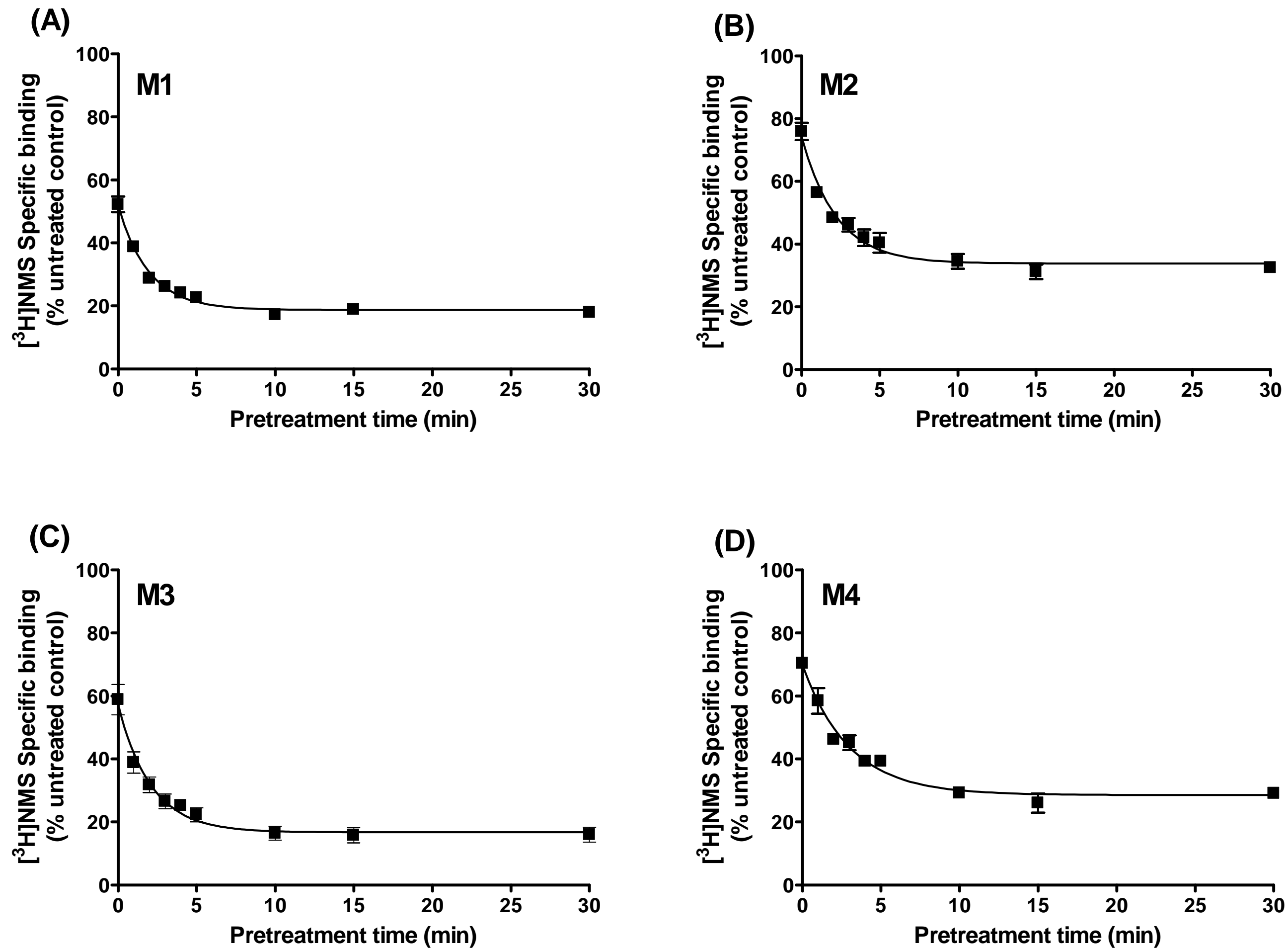


Figure 1. Time course of the development of xanomeline wash-resistant binding at hM1-hM4 muscarinic receptor subtypes expressed in CHO cells. Cells were treated with 10 μM xanomeline for increasing lengths of time (< 15 seconds to 30 minutes) followed by washing and incubation with 0.2 nM [^{3}H]NMS for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Binding in the absence of xanomeline pretreatment is denoted by 100%. Individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 7400, 7600, 5700 and 9900 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Figure 2

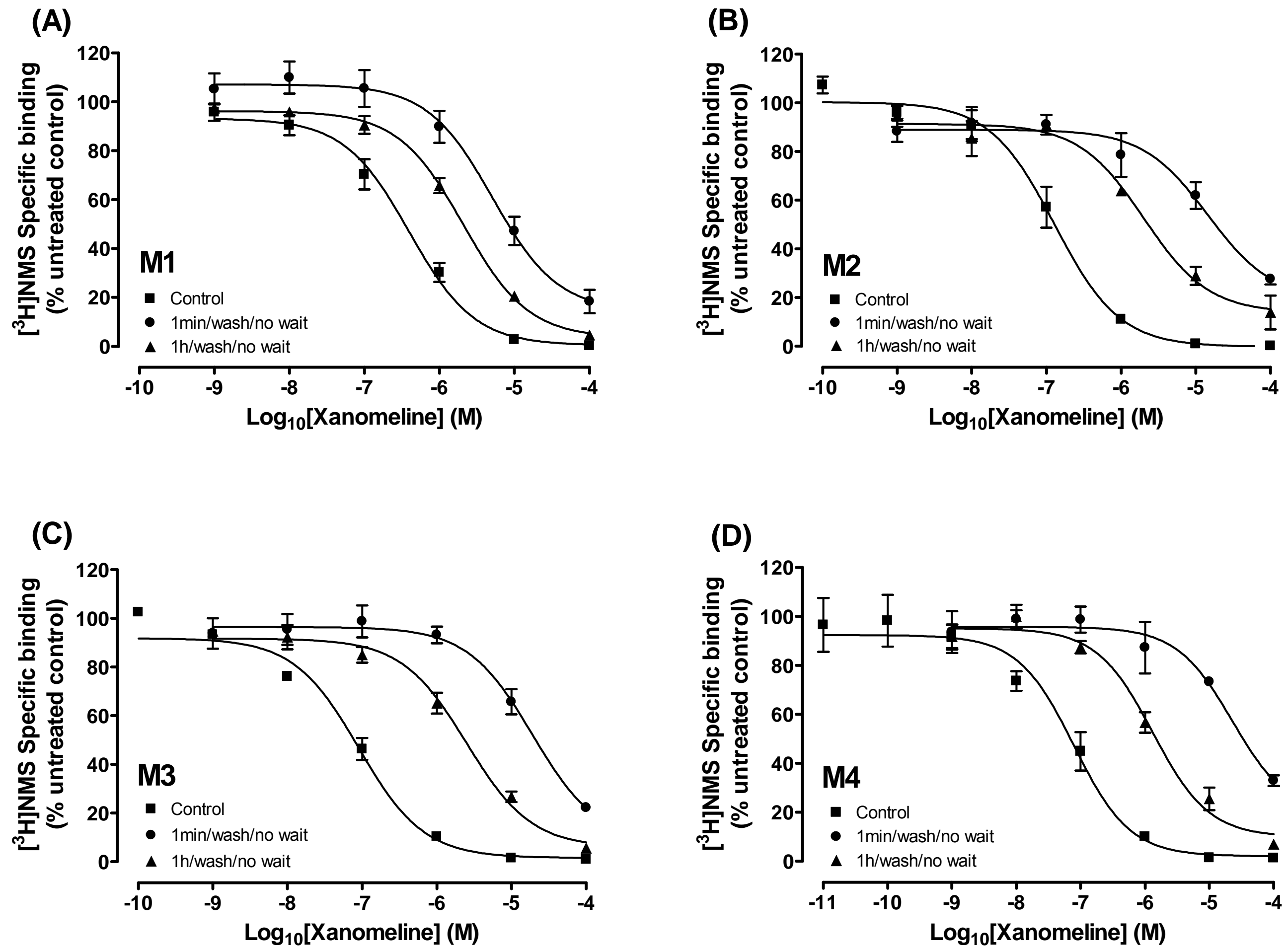


Figure 2. Inhibition of [^{3}H]NMS binding for acute xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of xanomeline for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing. Subsequently, cells were incubated for 1 hour at 37°C with 0.2 nM [^{3}H]NMS concurrently with xanomeline (closed squares) or following xanomeline pretreatments. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 13200, 12100, 21300 and 19000 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 3-9 experiments conducted in triplicate.

Figure 3

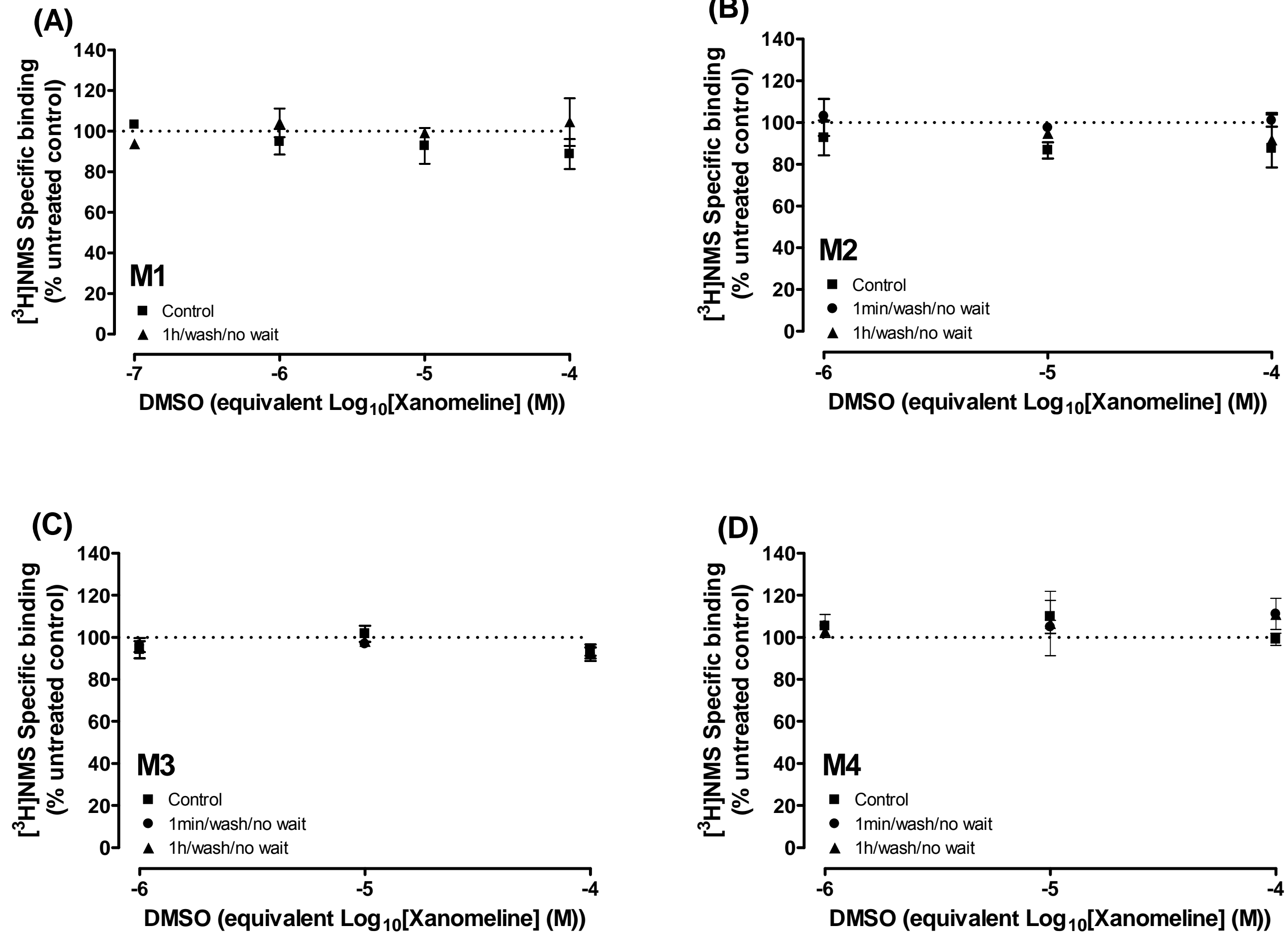


Figure 3. Effects of acute DMSO pretreatments on the inhibition of [^{3}H]NMS binding in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of DMSO in media (corresponding to the concentrations used in the preparation of xanomeline) for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing. Subsequently, cells were exposed concurrently to DMSO and 0.2 nM [^{3}H]NMS (closed squares) or to [^{3}H]NMS following DMSO pretreatments for 1 hour at 37ºC. A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control binding in the absence of DMSO treatment which had a mean of 11900, 15800, 21300 and 15400 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate. Data from (A) provided by Marianne Grant.

Figure 4

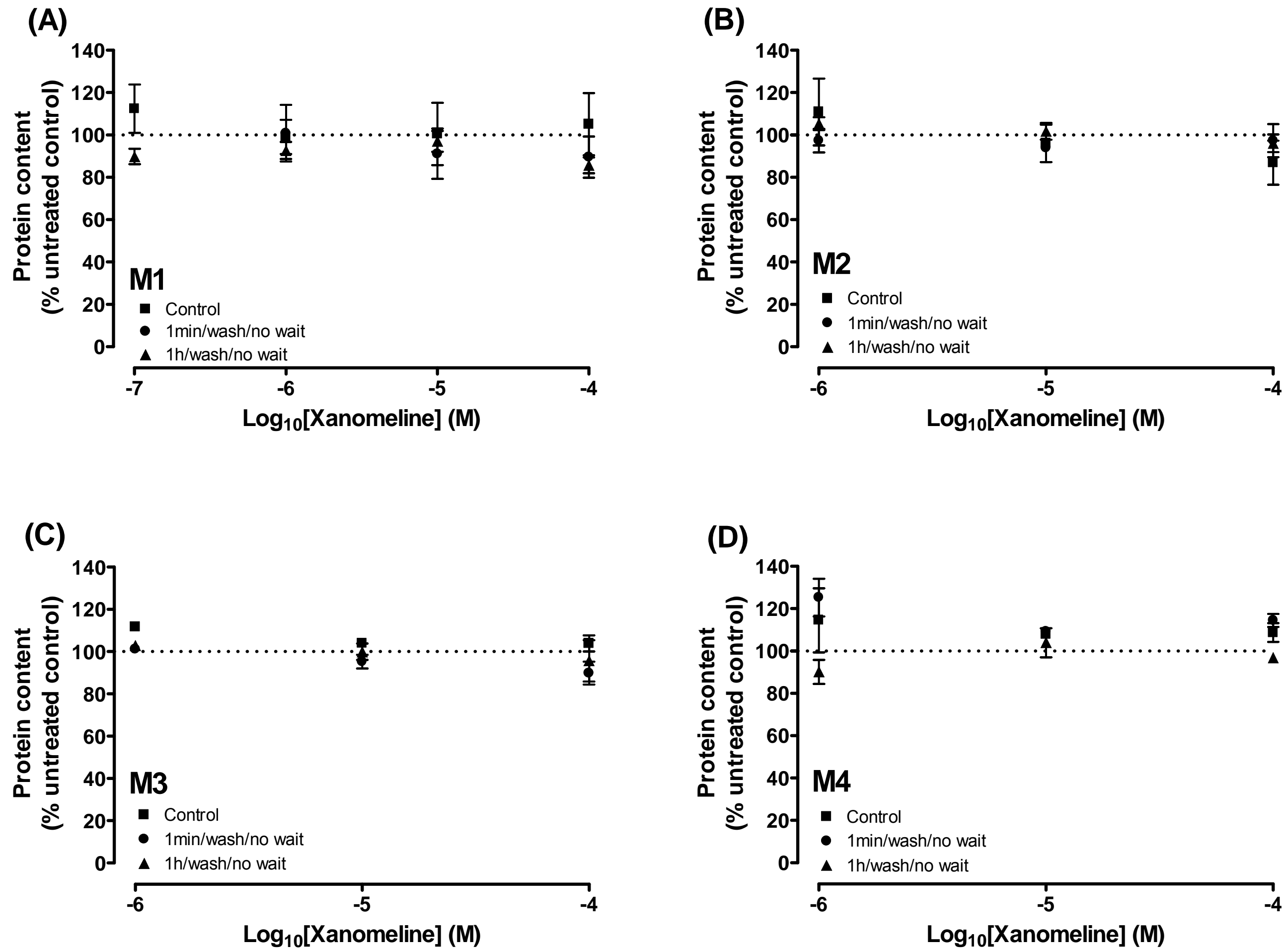


Figure 4. Effects of acute xanomeline pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of xanomeline for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing. Subsequently, reactions were terminated using 1 M NaOH and protein content on untreated cells incubated with xanomeline in HEPES buffer for 1 hour (closed squares) or xanomeline pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of xanomeline treatment which had a mean of 130, 256, 153 and 196 μg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2-3 experiments conducted in triplicate.

Figure 5

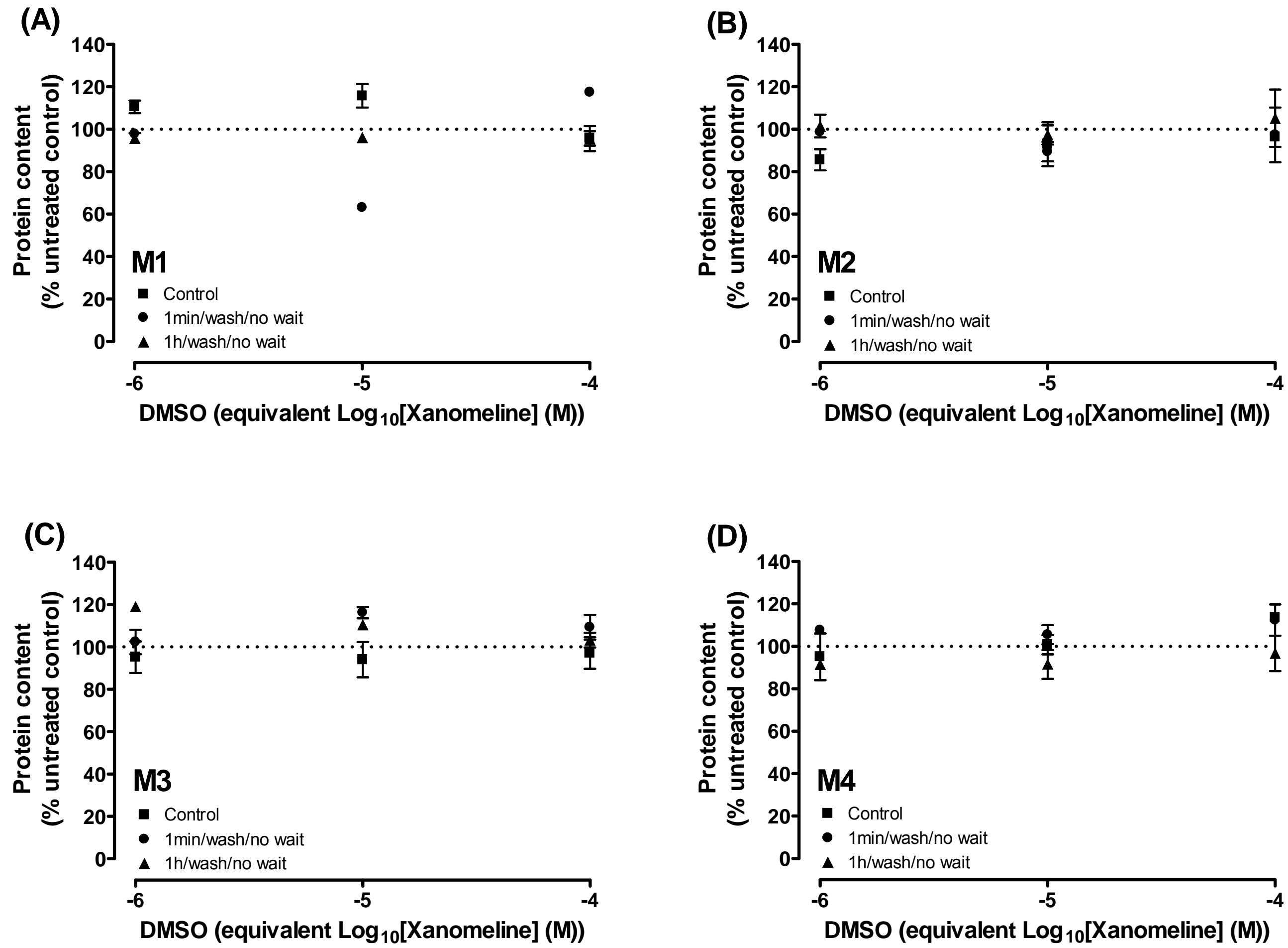


Figure 5. Effects of acute DMSO pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of DMSO in media (corresponding to the concentrations used in the preparation of xanomeline) for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing. Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with DMSO in HEPES buffer for 1 hour (closed squares) or DMSO pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of DMSO treatment which had a mean of 130, 265, 180 and 196 μg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 6

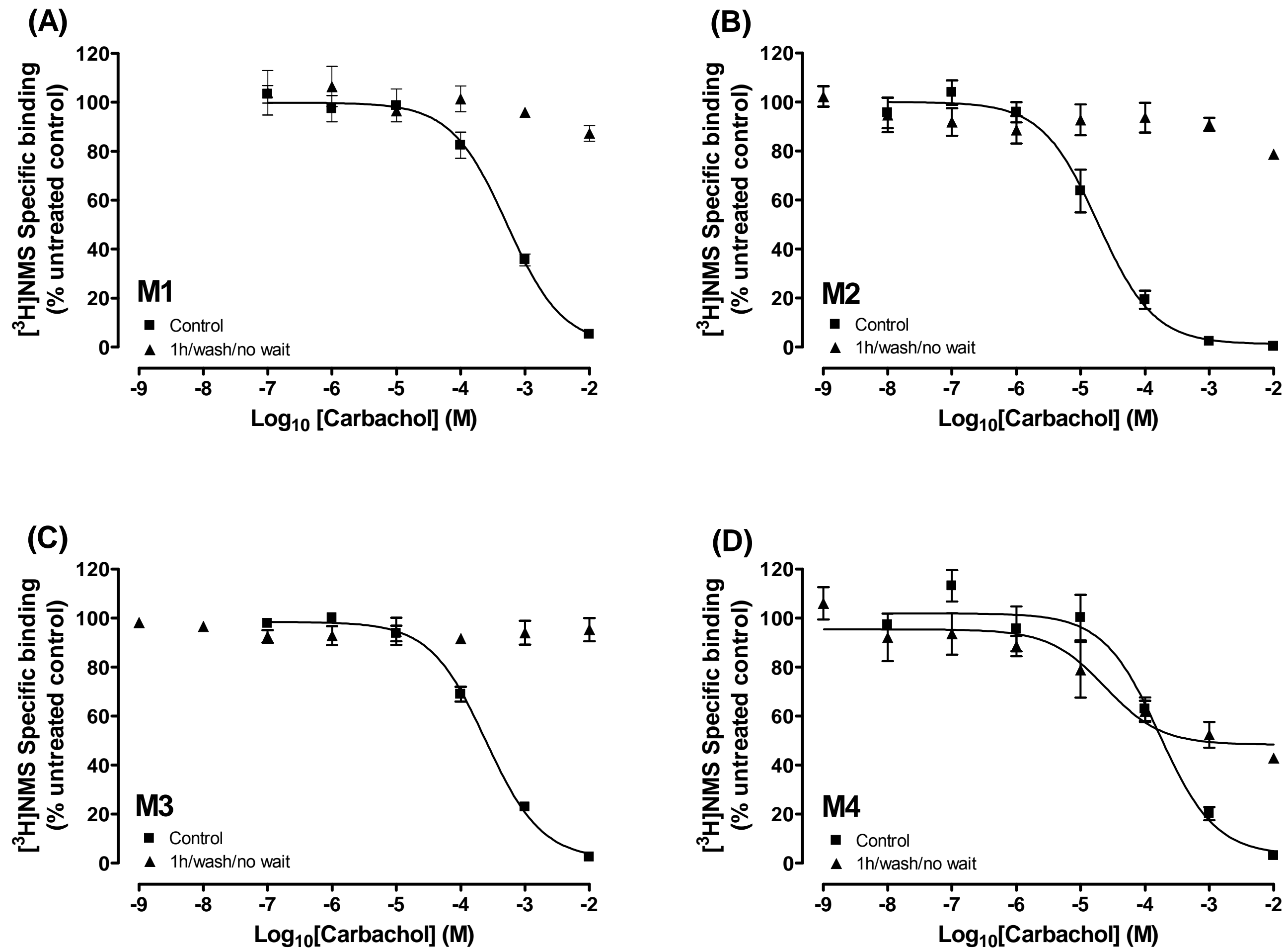


Figure 6. Inhibition of [^{3}H]NMS binding for acute carbachol pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of carbachol for 1 hour followed by washing (closed triangles). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with carbachol (closed squares) or following carbachol pretreatments for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Individual experiments were normalized to control binding in the absence of carbachol treatment which had a mean of 13000, 16500, 17700, and 16100 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate.

Figure 7

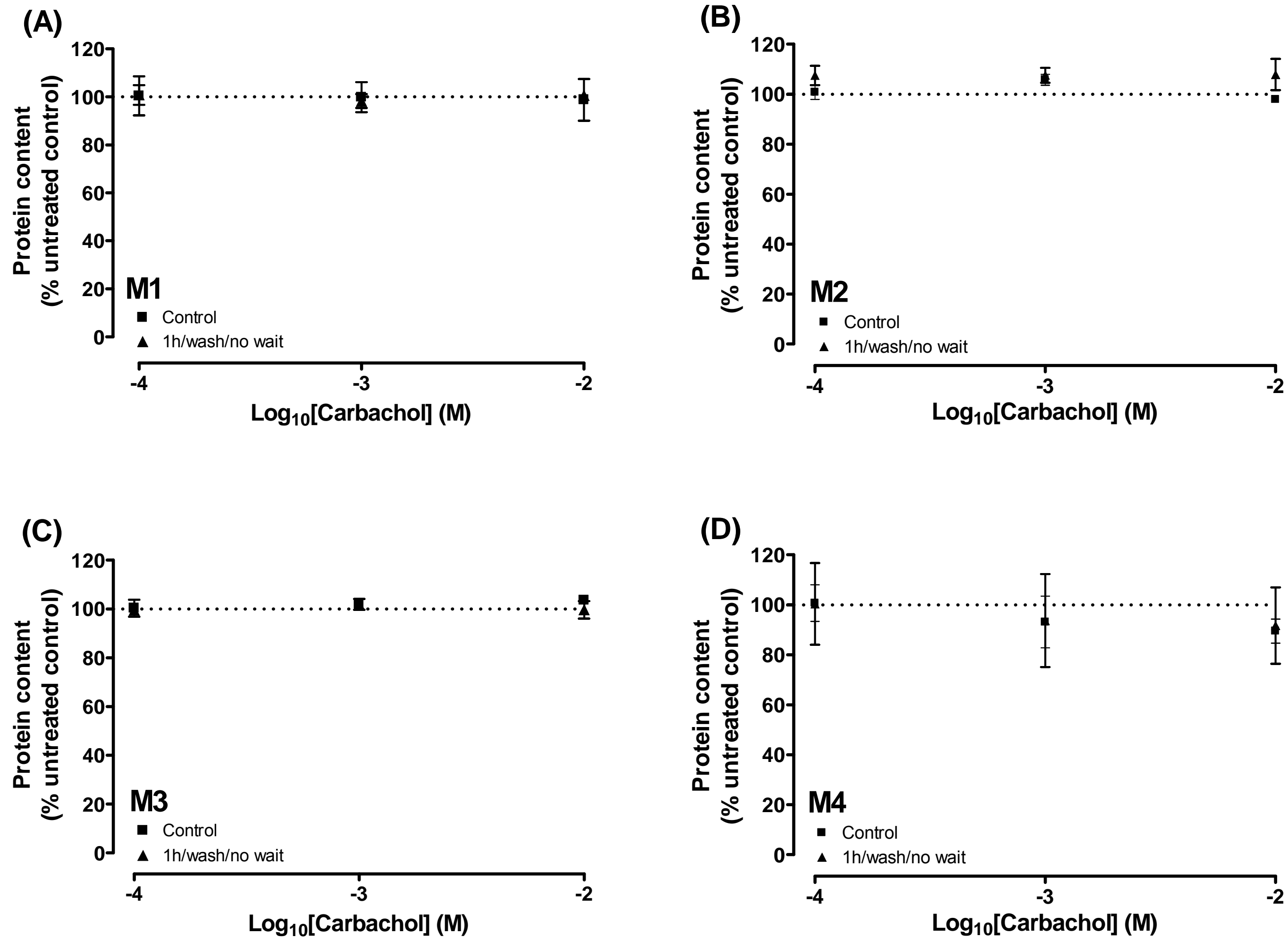


Figure 7. Effects of acute carbachol pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of carbachol for 1 hour (closed triangles) followed by washing. Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with carbachol in HEPES buffer for 1 hour (closed squares) or carbachol pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of carbachol treatment which had a mean of 180, 270, 153 and 296 µg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 8

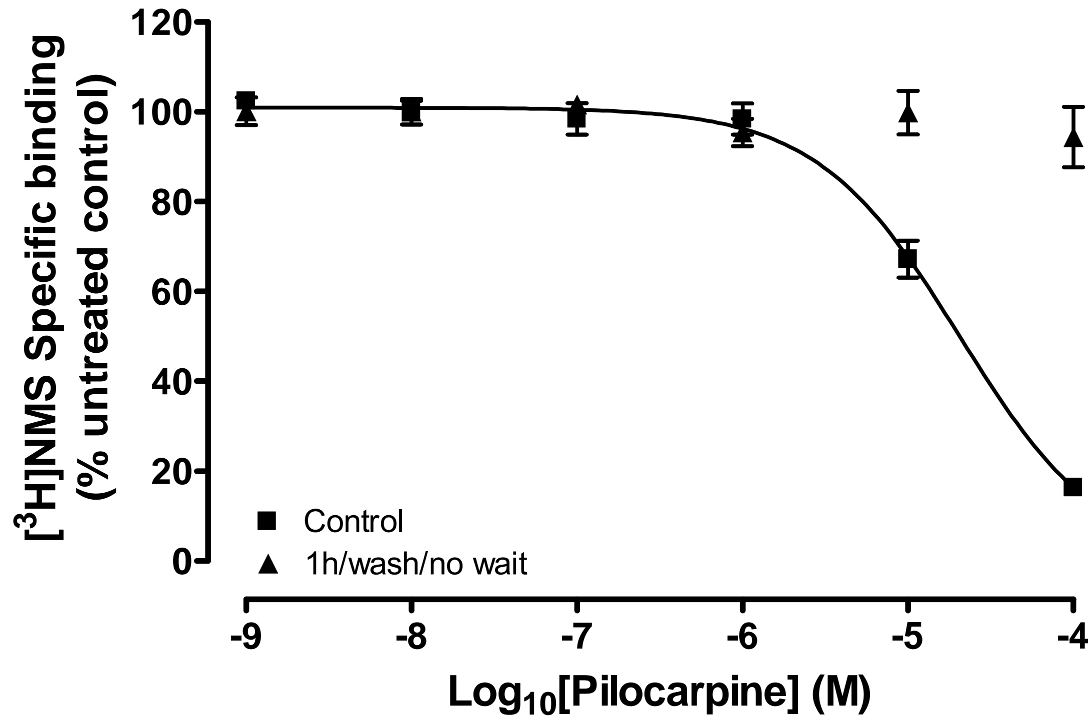


Figure 8. Inhibition of [^{3}H]NMS binding for acute pilocarpine pretreatments in CHO cells stably expressing the hM3 receptor subtype. Cells were pretreated with increasing concentrations of pilocarpine for 1 hour followed by washing (closed triangles). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with pilocarpine (closed squares) or following pilocarpine pretreatments for 1 hour at 37°C. Individual experiments were normalized to control binding in the absence of pilocarpine treatment which had a mean of 17500 dpm/well. Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Figure 9

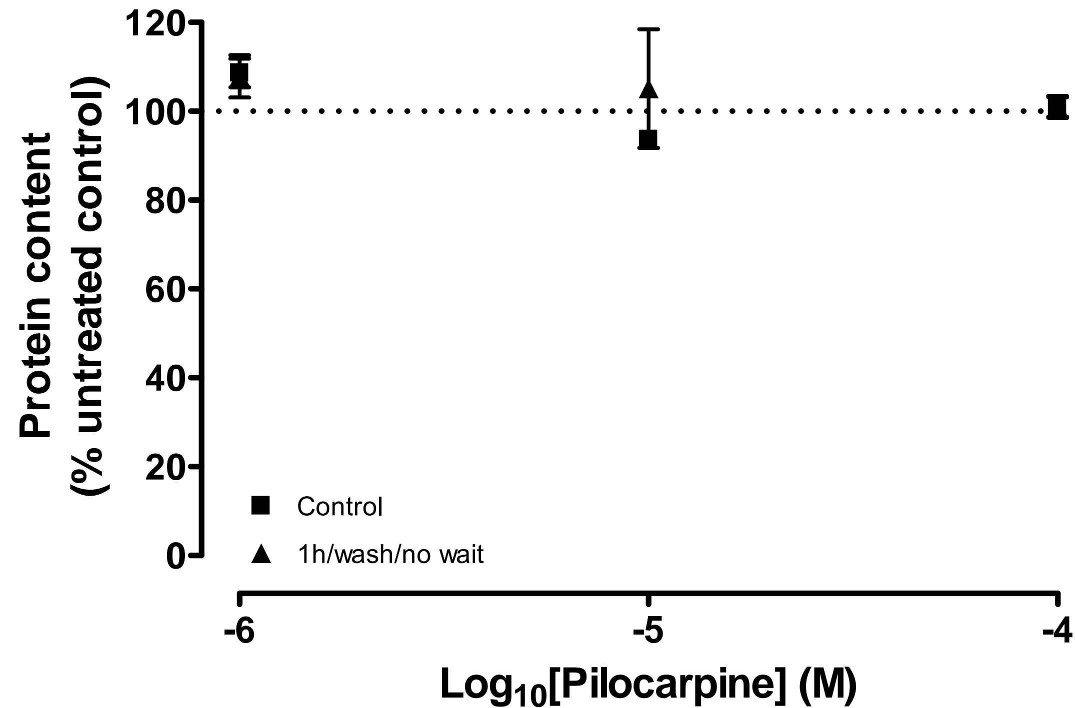


Figure 9. Effects of acute pilocarpine pretreatments on protein content in CHO cells stably expressing the hM3 receptor subtype. Cells were pretreated with increasing concentrations of pilocarpine for 1 hour (closed triangles) followed by washing. Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with pilocarpine in HEPES buffer for 1 hour (closed squares) or pilocarpine pretreated cells was determined using the method of Bradford (Bradford, 1976). Individual experiments were normalized to control protein in the absence of pilocarpine treatment which had a mean of 180 µg/well. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 10

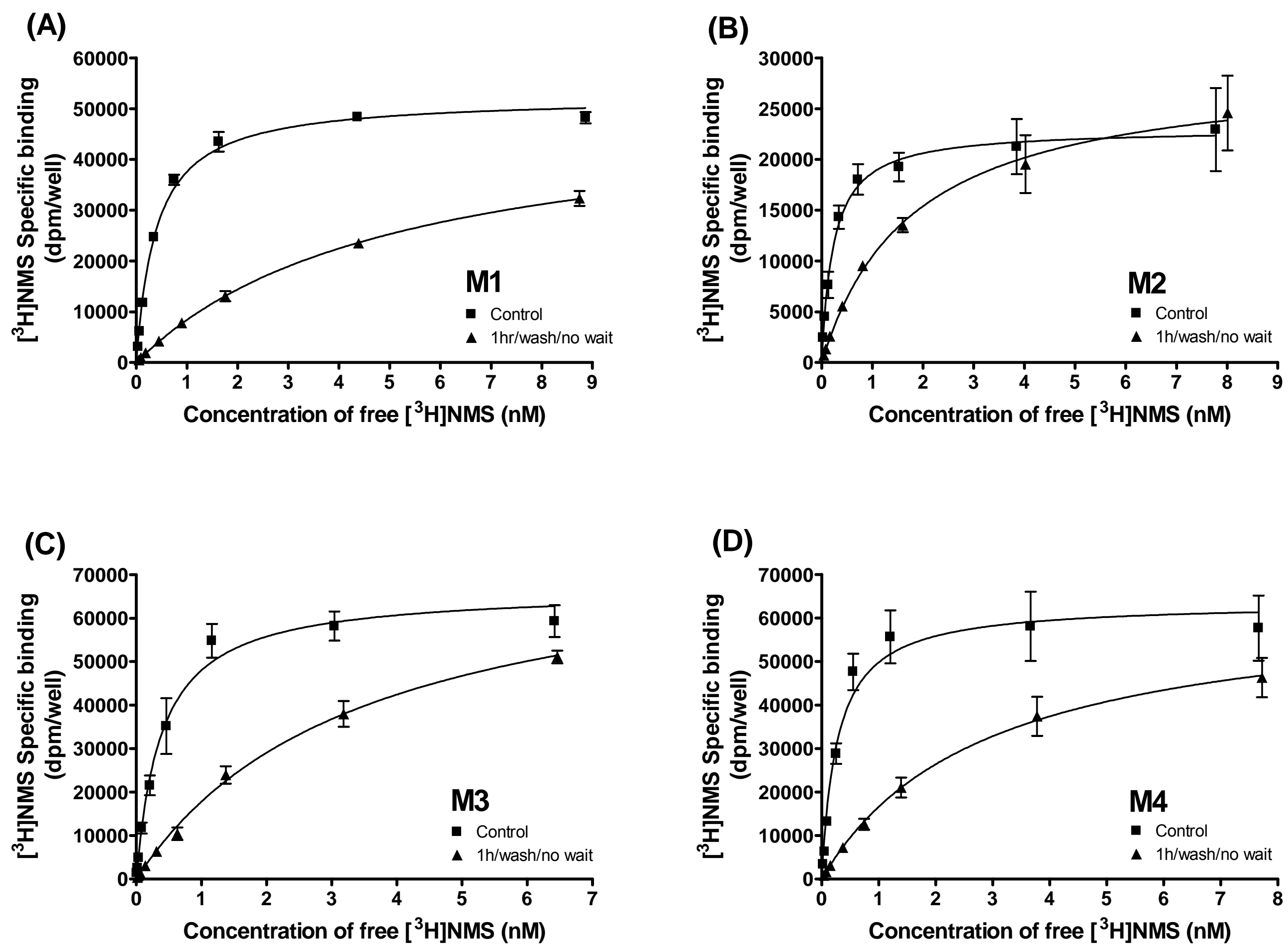


Figure 10. [^{3}H]NMS saturation binding subsequent to acute xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with 10 μM xanomeline for 1 hour followed by washing (closed triangles). Subsequently, untreated (closed squares) or xanomeline pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.01 – 8.9 nM) for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate.

Figure 11

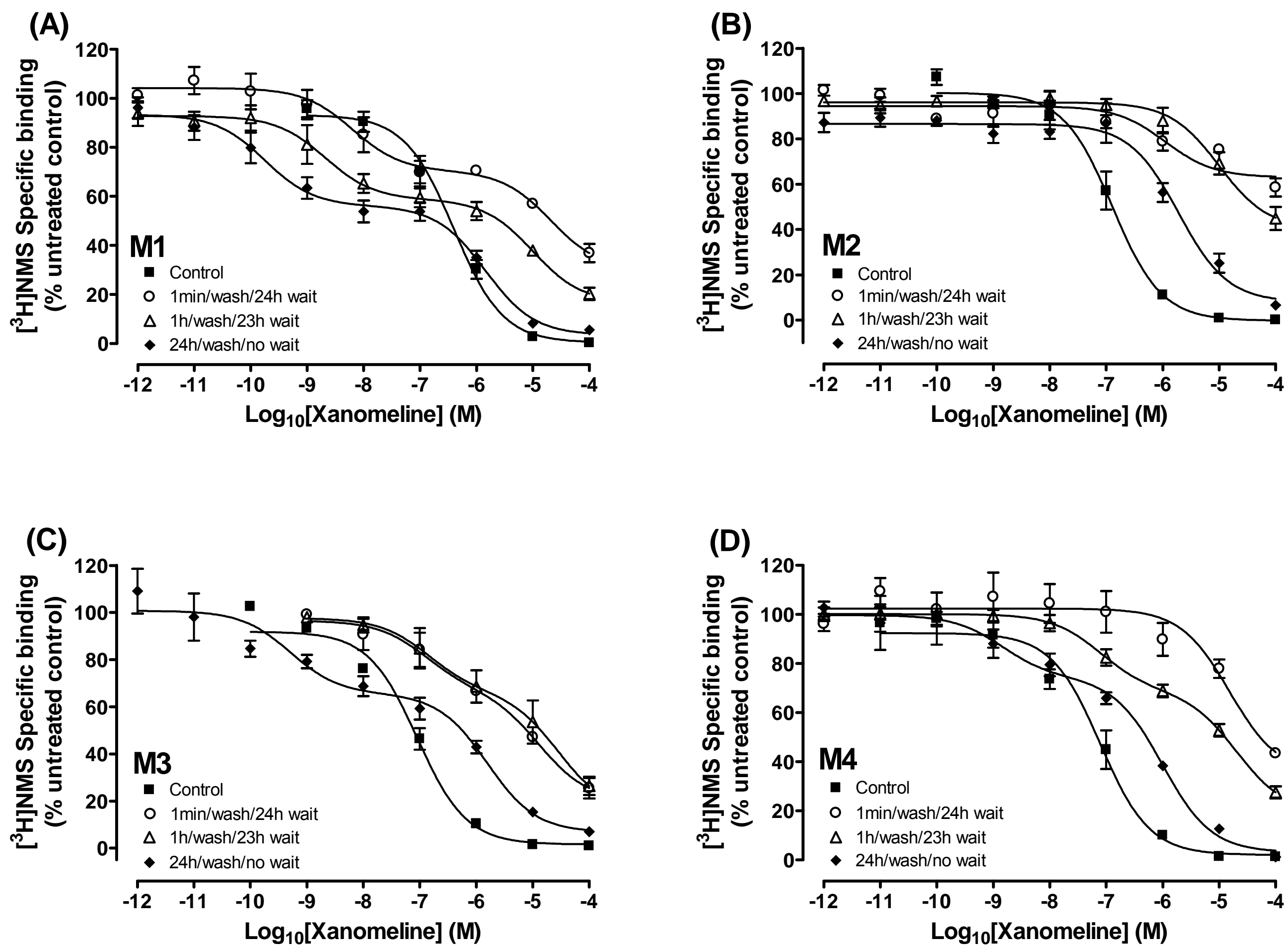


Figure 11. Inhibition of [^{3}H]NMS binding for long-term xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of xanomeline for 1 minute or 1 hour followed by washing and waiting 24 (open circles) or 23 (open triangles) hours, respectively. An additional group of cells was treated with xanomeline for 24 hours prior to washing (closed diamonds). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with xanomeline (closed squares) or following xanomeline pretreatments for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 13200, 12100, 21300 and 19000 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 3-9 experiments conducted in triplicate.

Figure 12

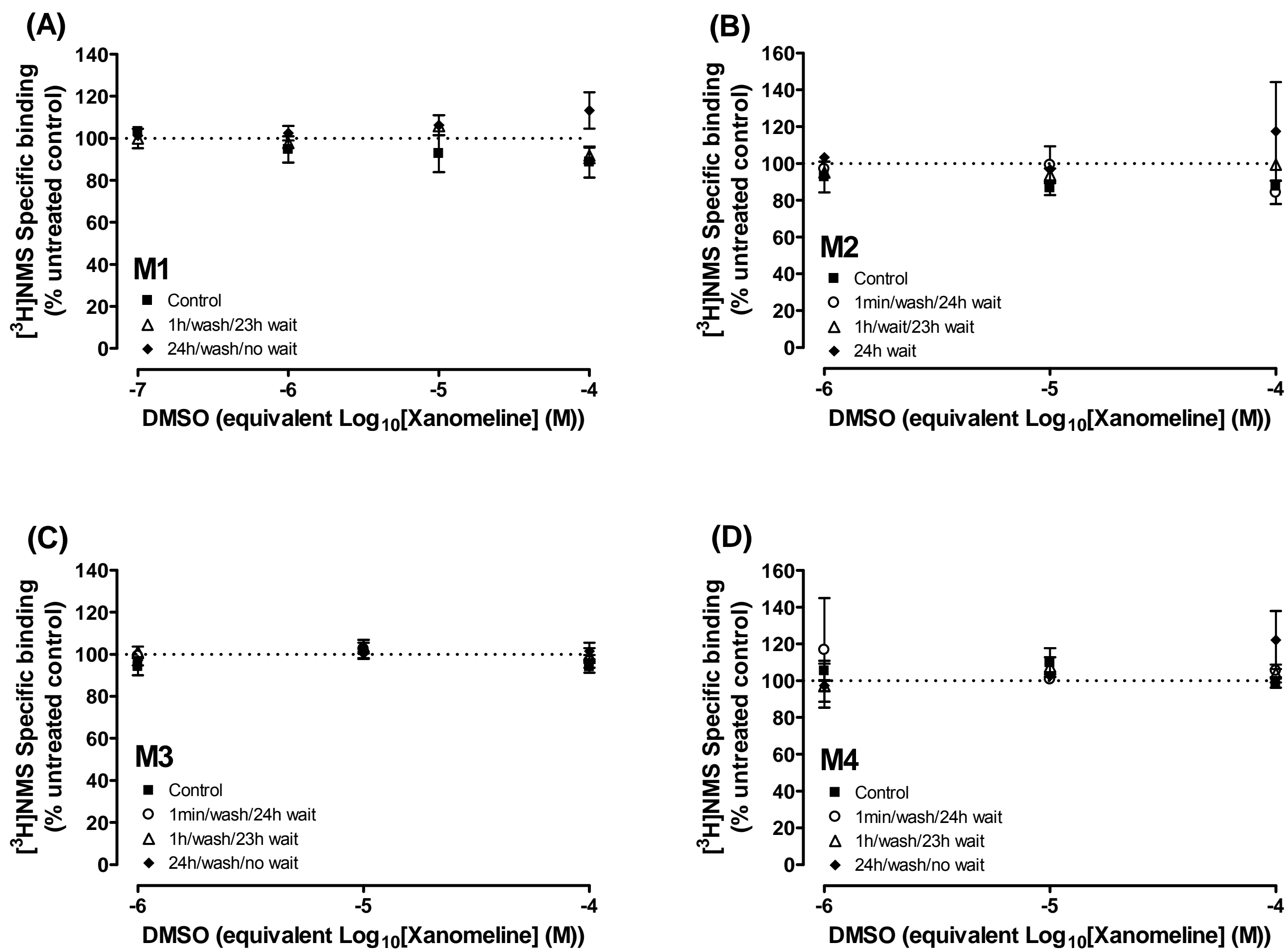


Figure 12. Effects of long-term DMSO pretreatments on the inhibition of [^{3}H]NMS binding in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of DMSO in media (corresponding to the concentrations used in the preparation of xanomeline) for 1 minute or 1 hour followed by washing and incubation in control media for 24 (open circles) or 23 (open triangles) hours, respectively. Additional cells were treated with DMSO for 24 hours prior to washing (closed diamonds). Subsequently, cells were exposed concurrently to DMSO and 0.2 nM [^{3}H]NMS (closed squares) or to [^{3}H]NMS following DMSO pretreatments for 1 hour at 37ºC. A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control binding in the absence of DMSO treatment which had a mean of 11900, 15800, 21300 and 15400 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate. Data from (A) provided by Marianne Grant.

Figure 13

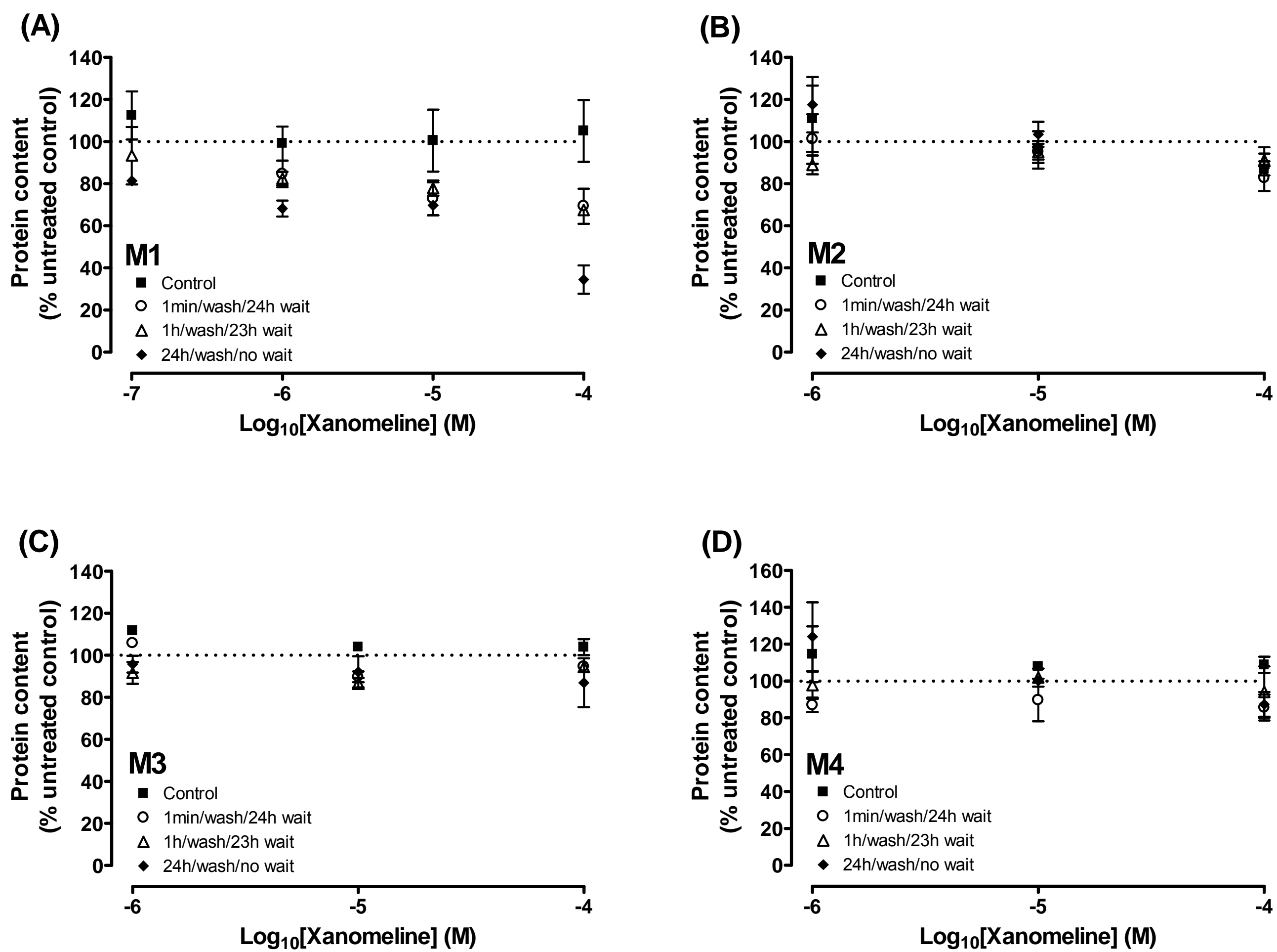


Figure 13. Effects of long-term xanomeline pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of xanomeline for 1 minute or 1 hour followed by washing and incubation in control media for 24 (open circles) or 23 (open triangles) hours, respectively. Additional cells were treated with xanomeline for 24 hours prior to washing (closed diamonds). Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with xanomeline in HEPES buffer for 1 hour (closed squares) or xanomeline pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of xanomeline treatment which had a mean of 130, 256, 153 and 196 μg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2-3 experiments conducted in triplicate.

Figure 14

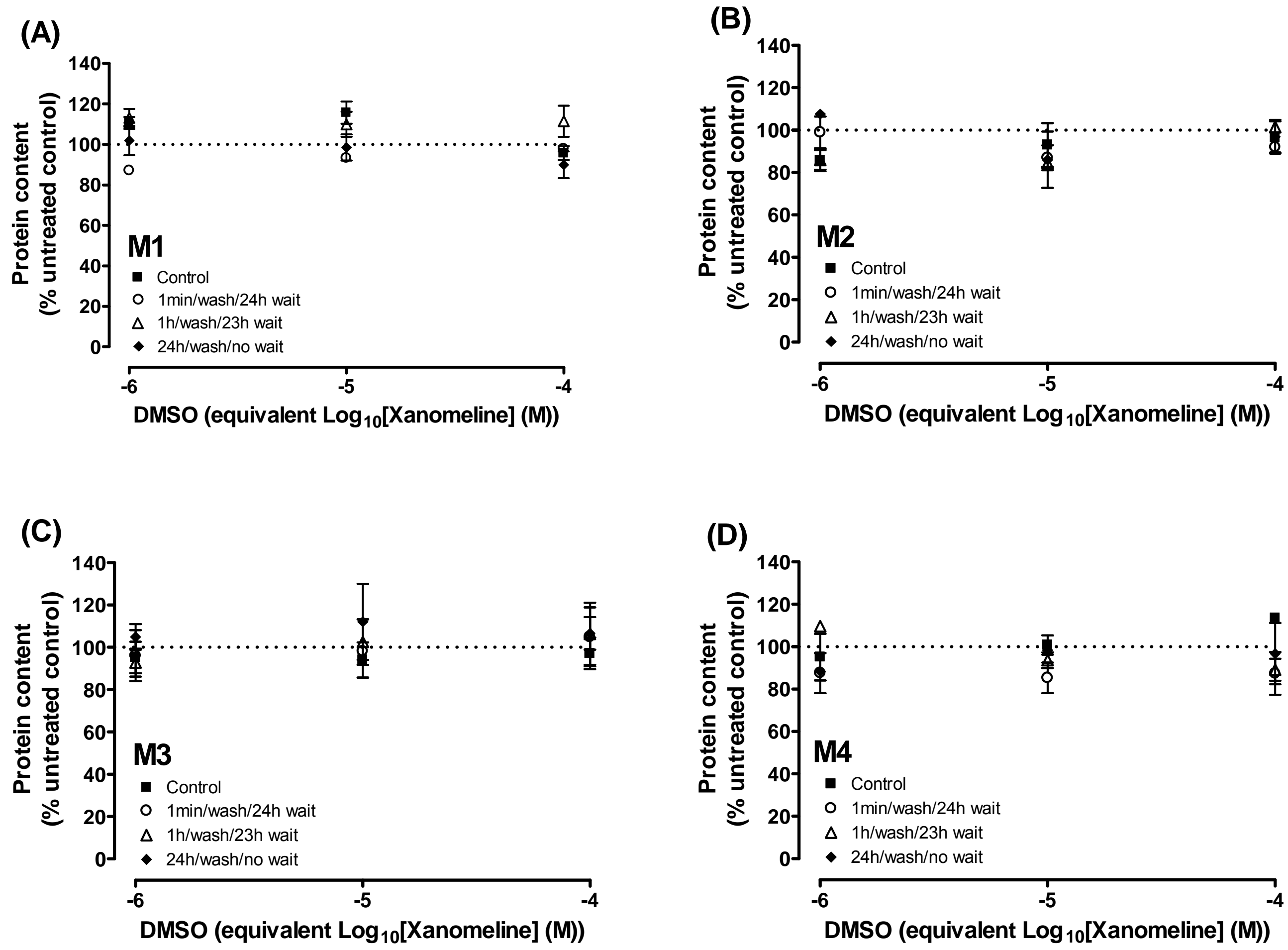


Figure 14. Effects of long-term DMSO pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of DMSO in media (corresponding to the concentrations used in the preparation of xanomeline) for 1 minute or 1 hour followed by washing and incubation for 24 (open circles) or 23 (open triangles) hours, respectively. Additional cells were treated with DMSO for 24 hours prior to washing (closed diamonds). Subsequently, reactions were terminated using 1 M NaOH and protein content on untreated cells incubated with DMSO in HEPES buffer (closed squares) or DMSO pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of DMSO treatment which had a mean of 130, 256, 180 and 196 μg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 15

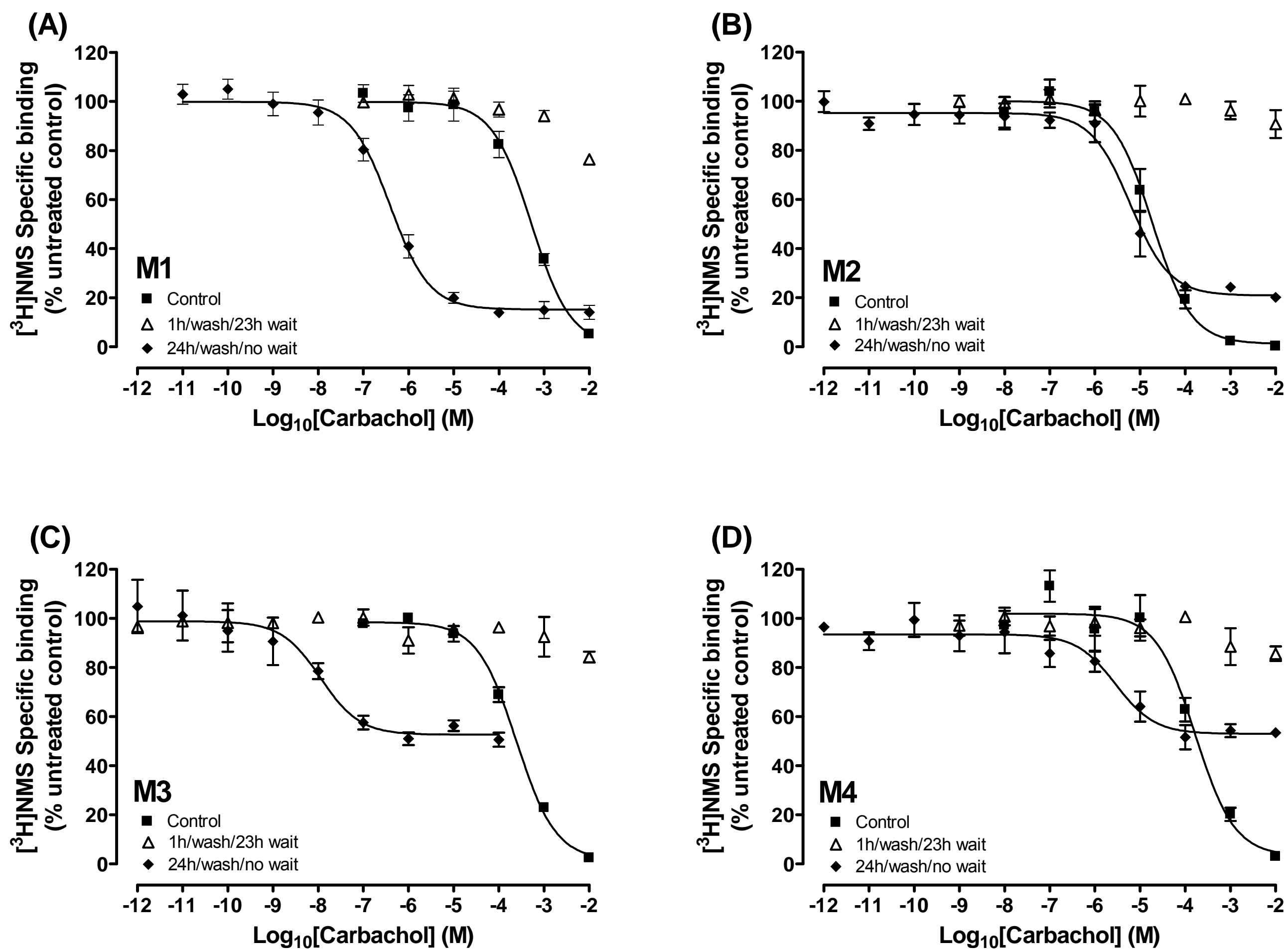


Figure 15. Inhibition of [^{3}H]NMS binding for long-term carbachol pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of carbachol for 1 hour followed by washing waiting 23 hours (open triangles). Additional cells were treated with carbachol for 24 hours prior to washing (closed diamonds). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with carbachol (closed squares) or following carbachol pretreatments for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Individual experiments were normalized to control binding in the absence of carbachol treatment which had a mean of 13000, 16500, 17700, and 16100 dpm/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 3-6 experiments conducted in triplicate.

Figure 16

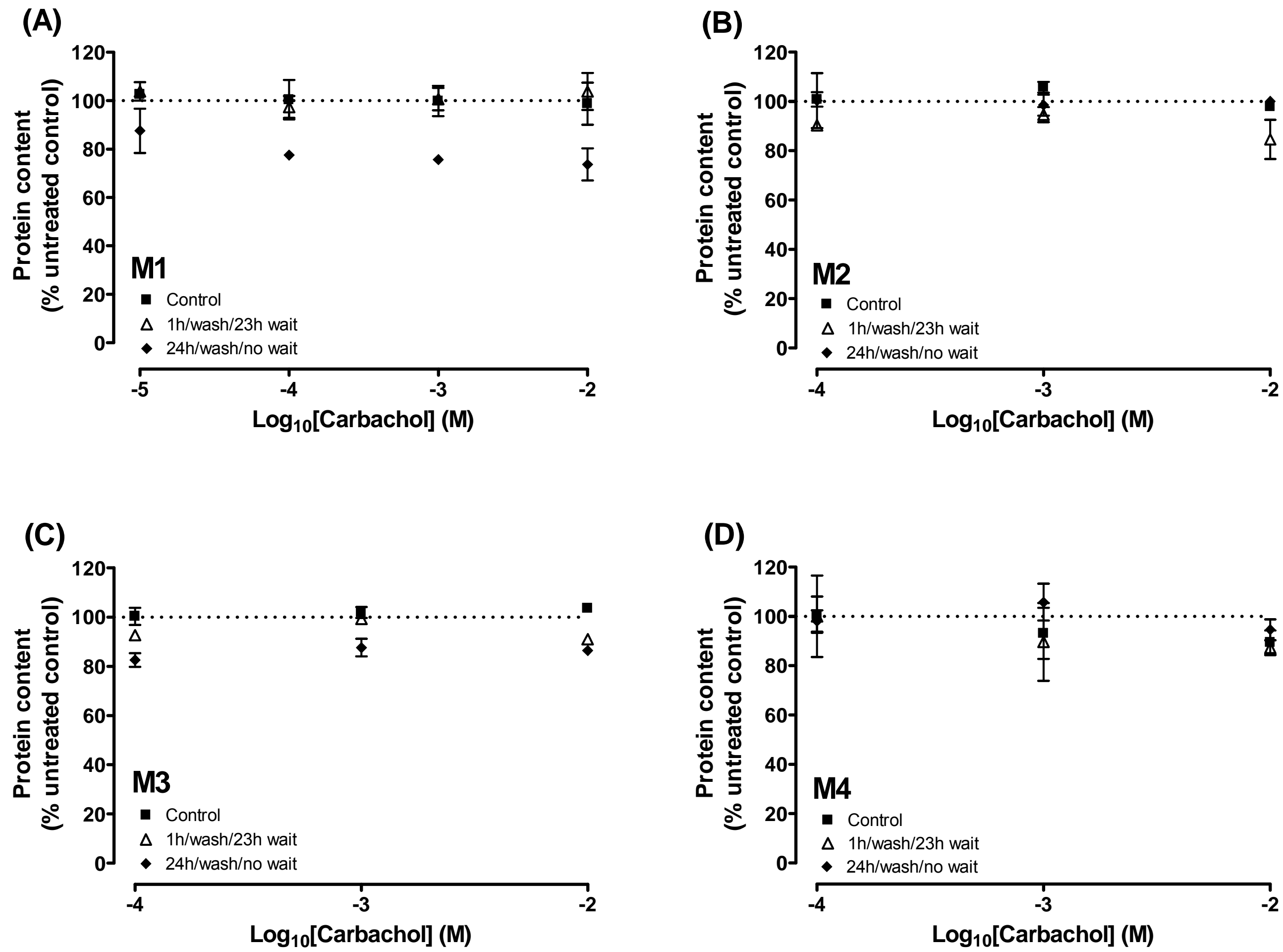


Figure 16. Effects of long-term carbachol pretreatments on protein content in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with increasing concentrations of carbachol for 1 hour followed by washing and incubation in control media for 23 hours (open triangles). Additional cells were treated with carbachol for 24 hours prior to washing (closed diamonds). Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with carbachol in HEPES buffer for 1 hour (closed squares) or carbachol pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of carbachol treatment which had a mean of 180, 270, 153 and 296 µg/well for M1, M2, M3 and M4 receptors, respectively. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 17

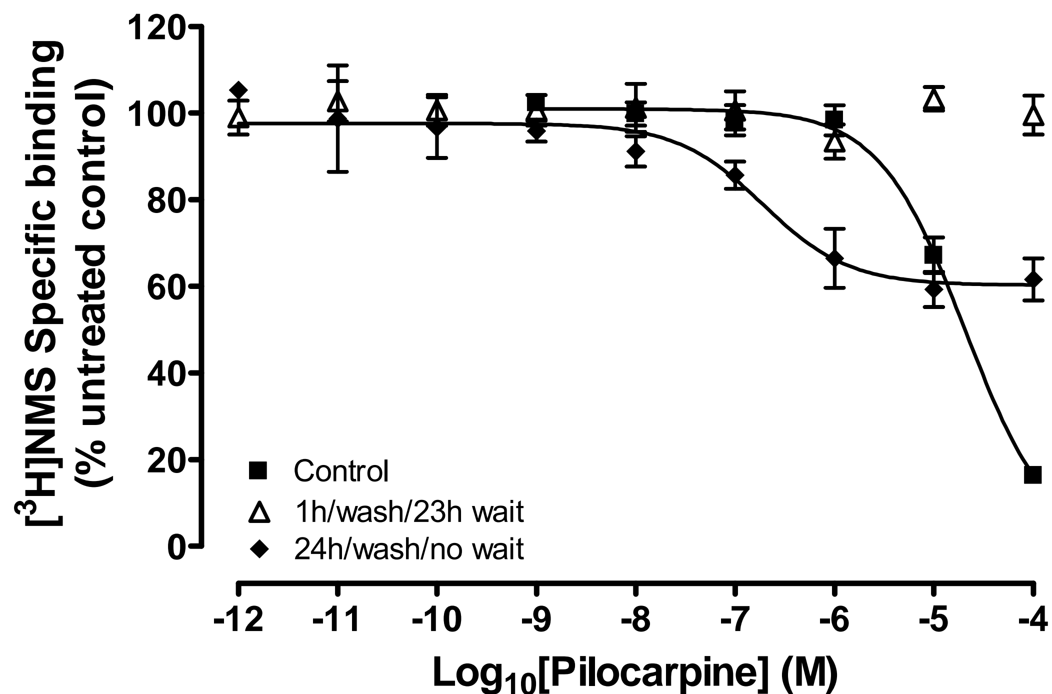


Figure 17. Inhibition of [^{3}H]NMS binding for long-term pilocarpine pretreatments in CHO cells stably expressing the hM3 receptor subtype. Cells were pretreated with increasing concentrations of pilocarpine for 1 hour followed by washing waiting 23 hours (open triangles). Additional cells were treated with pilocarpine for 24 hours prior to washing (closed diamonds). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with pilocarpine (closed squares) or following pilocarpine pretreatments for 1 hour at 37°C. Individual experiments were normalized to control binding in the absence of pilocarpine treatment which had a mean of 17500 dpm/well. Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Figure 18

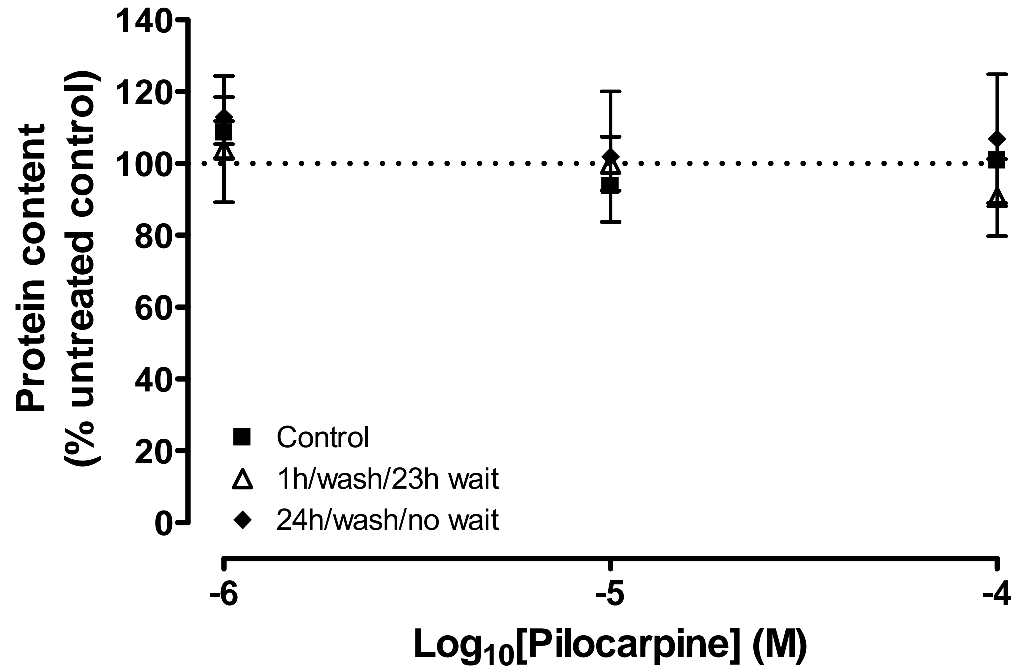
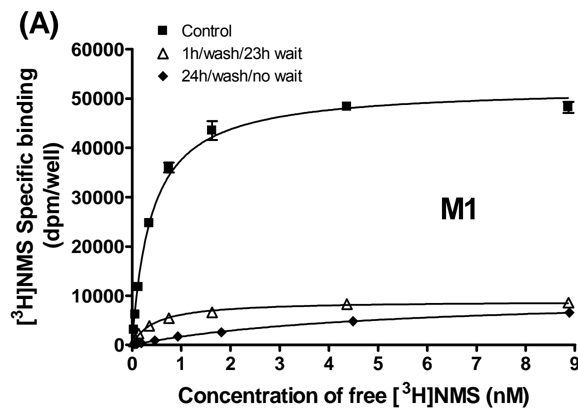
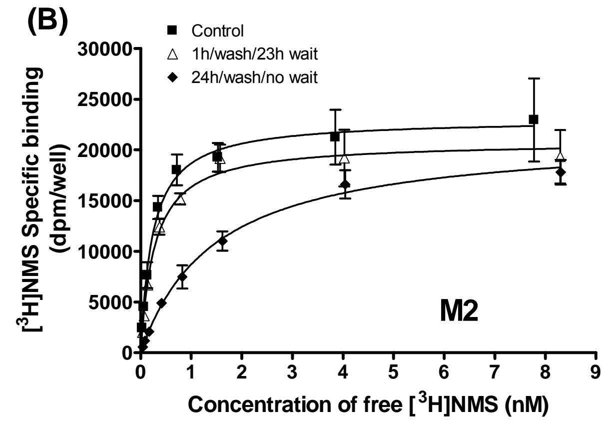
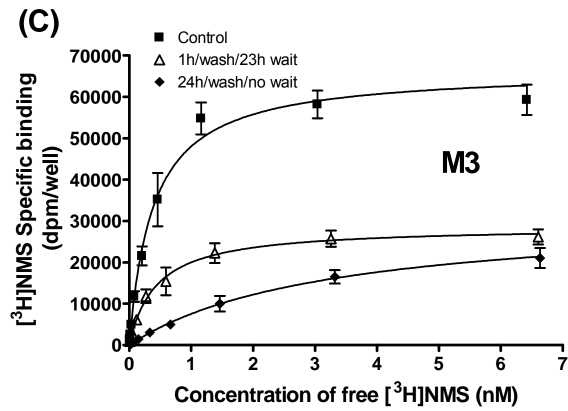


Figure 18. Effects of long-term pilocarpine pretreatments on protein content in CHO cells stably expressing hM3 receptor subtype. Cells were pretreated with increasing concentrations of pilocarpine for 1 hour followed by washing and incubation in control media for 23 hours (open triangles). Additional cells were treated with pilocarpine for 24 hours prior to washing (closed diamonds). Subsequently, reactions were terminated using 1 M NaOH. Protein content on untreated cells incubated with pilocarpine in HEPES buffer for 1 hour (closed squares) or pilocarpine pretreated cells was determined using the method of Bradford (Bradford, 1976). A) M1 receptor, B) M2 receptor, C) M3 receptor and D) M4 receptor. Individual experiments were normalized to control protein in the absence of pilocarpine treatment which had a mean of 180 µg/well. Values represent the means ± S.E. of 2 experiments conducted in triplicate.

Figure 19







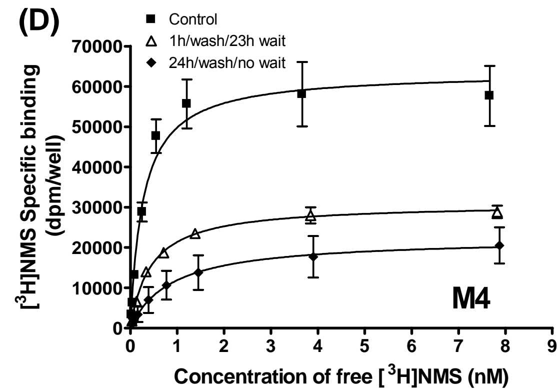


Figure 19. [^{3}H]NMS saturation binding subsequent to long-term xanomeline pretreatments in CHO cells stably expressing hM1-hM4 receptor subtypes. Cells were pretreated with 10 μM xanomeline for 1 hour followed by washing and waiting 23 hours (open triangles). Additional cells were treated with xanomeline for 24 hours prior to washing (closed diamonds). Subsequently, untreated (closed squares) or xanomeline pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.01 – 8.9 nM) for 1 hour at 37°C. A) M1 receptor, B) M2 receptor, C) M3 receptor, and D) M4 receptor. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate.

Figure 20

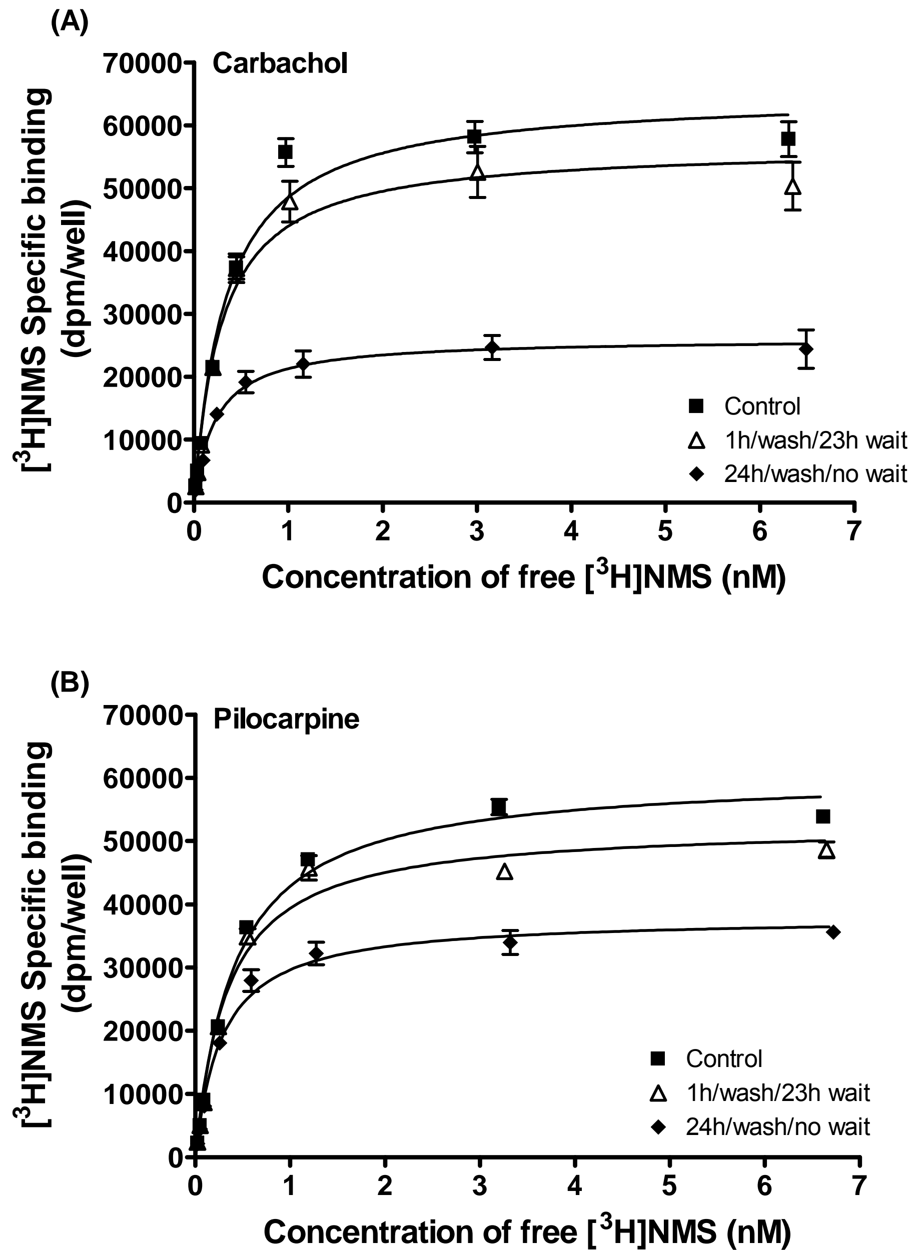


Figure 20. [^{3}H]NMS saturation binding subsequent to long-term agonist pretreatments in CHO cells stably expressing the hM3 receptor subtype. Cells expressing the M3 receptor were exposed to 10 µM agonist for 1 hour followed by washing and waiting 23 hours (open triangles). Additional cells were exposed to agonist for 24 hours prior to washing (closed diamonds). Subsequently, untreated (closed squares) or agonist pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.02 – 6.7 nM) for 1 hour at 37ºC. A) Carbachol and B) Pilocarpine. Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Table 1. Short- and long-term effects of xanomeline, carbachol or pilocarpine binding on [^{3}H]NMS binding in CHO cells expressing hM1-hM4 receptor subtypes. Cells were treated with increasing concentrations of xanomeline (1 pM – 100 µM), carbachol (1 pM – 10 mM) or pilocarpine (1 pM – 100 µM) for 1 minute or 1 hour followed by washing and immediate use in the binding assay or subsequent to incubation for 24 or 23 hours, respectively, in the absence of free agonist. Additional groups of cells were treated with increasing concentrations of agonists for 24 hours prior to washing. Pretreated and washed cells in all groups were incubated with 0.2nM [^{3}H]NMS at 37ºC for 1 hour. Control untreated cells were incubated simultaneously with agonist and 0.2nM [^{3}H]NMS during the binding assay. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	M1				M2	
	pIC_{50} [a]	pIC_{50} – H [b]	pIC_{50} – L [c]	I_{max} [d]	pIC_{50}	I_{max}
Xanomeline						
Control (untreated) [e]	6.5 ± 0.12			99 ± 0.3	6.9 ± 0.17	100 ± 0.3
1 min/washout/no wait	5.4 ± 0.13 [$]			85 ± 5.2 [‡]	5.0 ± 0.29 [$]	80 ± 6.0 [‡]
1 min/washout/24 h wait		8.2 ± 0.21 [†] (47 ± 4.3%)	4.6 ± 0.11 [†]	74 ± 6.2 [‡]	5.9 ± 0.44	41 ± 5.3 [‡]
1 h/washout/no wait	5.7 ± 0.05 [$]			97 ± 0.8	5.7 ± 0.12 [$]	87 ± 6.9
1 h/washout/23 h wait		8.6 ± 0.21* (43 ± 2.4%)	5.0 ± 0.06*	84 ± 2.8 [‡]	5.0 ± 0.15*	59 ± 3.0 [‡]
24 h/washout/no wait		9.8 ± 0.21*[†] (43 ± 3.7 %)	5.8 ± 0.06	97 ± 0.6	5.7 ± 0.12	93 ± 1.6
Carbachol						
Control (untreated)	3.3 ± 0.01			99 ± 0.3	4.7 ± 0.13	99 ± 0.8
1 h/washout/no wait	ND			ND	ND	ND
1 h/washout/23 h wait	ND			ND	ND	ND
24 h/washout/no wait	6.3 ± 0.08 [#]			88 ± 1.4 [‡]	5.2 ± 0.17	80 ± 1.4 [‡]

Table 1 cont.

	M3				M4			
	pIC_{50}	**pIC_{50} - H**	**pIC_{50} - L**	**I_{max}**	**pIC_{50}**	**pIC_{50} - H**	**pIC_{50} - L**	**I_{max}**
Xanomeline								
Control (untreated)[e]	7.1 ± 0.10			99 ± 0.4	7.1 ± 0.15			99 ± 0.7
1 min/washout/no wait	4.7 ± 0.08[$]			93 ± 3.0	4.6 ± 0.18[$]			85 ± 6.2[‡]
1 min/washout/24 h wait		7.7 ± 0.75[†] (40 ± 4.3%)	4.8 ± 0.43	82 ± 9.6[‡]	4.8 ± 0.12			68 ± 3.1[‡]
1 h/washout/no wait	5.6 ± 0.07[$]			95 ± 0.9	5.9 ± 0.05[$]			90 ± 2.6
1 h/washout/23 h wait		6.8 ± 0.39 (32 ± 9.9%)	4.3 ± 0.48	89 ± 5.5		7.1 ± 0.15* (38 ± 2.7%)	4.6 ± 0.10*	82 ± 1.1[‡]
24 h/washout/no wait		9.0 ± 0.46*[†] (41 ± 3.4 %)	5.7 ± 0.06	94 ± 0.8		8.6 ± 0.29*[†] (29 ± 1.7 %)	5.9 ± 0.04[†]	97 ± 0.6
Carbachol								
Control (untreated)	3.6 ± 0.05			98 ± 0.6	3.8 ± 0.05			97 ± 0.9
1 h/washout/no wait	ND			ND	4.7 ± 0.44			53 ± 4.5[‡]
1 h/washout/23 h wait	ND			ND	ND			ND
24 h/washout/no wait	7.8 ± 0.19[#]			48 ± 1.3[‡]	5.5 ± 0.18[#]			46 ± 2.7[‡]
Pilocarpine								
Control (untreated)	4.7 ± 0.11			99 ± 0.9				
1 h/washout/no wait	ND			ND				
1 h/washout/23 h wait	ND			ND				
24 h/washout/no wait	6.7 ± 0.05[†]			40 ± 5.4[‡]				

Table 1 cont.

[a] Negative logarithm of the IC_{50} for binding to a single potency site.

[b] Negative logarithm of the IC_{50} for the high-potency site. Percent of binding sites shown in parentheses.

[c] Negative logarithm of the IC_{50} for the low-potency site.

[d] Maximal percent inhibition of [^{3}H]NMS binding.

[e] Control cells were incubated simultaneously with agonist and radioligand in the binding assay.

ND – parameters were not determined

[$] Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} between either the xanomeline 1min/wash/no wait or 1h/wash/no wait group compared to control.

[†] ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} where indicated between xanomeline 1min/wash/24h wait or 24h/wash/no wait pretreated groups compared to xanomeline 1min/wash/no wait treatment.

* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} where indicated between xanomeline 1h/wash/23h wait or 24h/wash/no wait pretreated groups compared to xanomeline 1h/wash/no wait treatment.

[#] Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} between carbachol 24h/wash/no wait compared to carbachol control.

[±] Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} between pilocarpine 24h/wash/no wait compared to pilocarpine control.

[‡] Significantly different ($p < 0.5$) from 100 percent as determined by ANOVA followed by Dunnett's post-test comparison.

Table 2. [^{3}H]NMS saturation binding parameters for xanomeline, carbachol or pilocarpine pretreatment and waiting periods in CHO cells expressing hM1-hM4 receptor subtypes. Cells were treated with 10 μM xanomeline, carbachol or pilocarpine for 1 hour followed by washing and immediate use in the binding assay or subsequent to incubation for 23 hours in the absence of free agonist. An additional group of cells was treated with agonist for 24 hours prior to washing. Untreated control or agonist pretreated cells were then incubated with increasing concentrations of [^{3}H]NMS (0.01 – 8.9 nM) for 1 hour at 37ºC. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	M1		M2		M3		M4	
	K_d [a]	B_{max} [b]	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
Xanomeline								
Control (untreated)	0.39 ± 0.02	52300 ± 700	0.24 ± 0.08	23200 ± 3200	0.42 ± 0.06	67700 ± 3200	0.27 ± 0.03	63600 ± 8400
1 h/washout/no wait	5.01 ± 0.18*	51000 ± 2500	1.42 ± 0.31*	26500 ± 5100	3.52 ± 0.52*	78500 ± 1600	2.89 ± 0.18*	64100 ± 5900
1 h/washout/23h wait	0.64 ± 0.10	9300 ± 300*	0.27 ± 0.05	20900 ± 2800	0.59 ± 0.21	30400 ± 2000*	0.43 ± 0.01	30400 ± 1200*
24 h/washout/no wait	5.11 ± 0.80*	10300 ± 1100*	1.47 ± 0.16*	21500 ± 1500	4.19 ± 0.93*	37000 ± 4600*	2.71 ± 0.26*	32900 ± 6500*
Carbachol								
Control (untreated)					0.34 ± 0.01	65000 ± 2900		
1 h/washout/23h wait					0.28 ± 0.01	56600 ± 4200		
24 h/washout/no wait					0.23 ± 0.03†	26200 ± 2700†		
Pilocarpine								
Control (untreated)					0.42 ± 0.03	60600 ± 960		
1 h/washout/23h wait					0.34 ± 0.02	52600 ± 850$		
24 h/washout/no wait					0.28 ± 0.01$	38000 ± 1500$		

Table 2 cont.
[a] Equilibrium dissociation constant for [^{3}H]NMS binding.
[b] Cell surface receptor density (dpm/well)
*ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in K_d or B_{max} between the xanomeline pretreated groups compared with their untreated control for each of the individual subtypes.
† ANOVA followed by Dunnett's post-test detected a significant difference ($p < 0.05$) in K_d and B_{max} between carbachol pretreated groups compared to carbachol control.
$ ANOVA followed by Dunnett's post-test detected a significant difference ($p < 0.05$) in K_d and B_{max} between pilocarpine treated groups compared to pilocarpine control.

CHAPTER 4: Determination of the acute and long-term functional effects of xanomeline at the M1 and M3 muscarinic receptors

Introduction

Muscarinic receptors are part of the G protein-coupled superfamily of receptors. Binding of an agonist to the receptor is thought to change the interaction of transmembrane domains 3, 5, 6 and 7 leading to the activation of down-stream signaling cascades (Eglen, 2005). This includes activation of phospholipase C, modulation of ion channels, activation of mitogen-activated protein kinase (involved in cell survival, differentiation and synaptic plasticity) and small GTPases (Volpicelli & Levey, 2004). Muscarinic M1 and M3 receptors are coupled to $G_{q/11}$ G proteins through the third cytoplasmic domain. One way that the stimulation of the M1 or M3 receptors can be assessed is by determining the production of radiolabeled inositol phosphates following loading of the cells with radiolabeled *myo*-inositol (Berridge et al., 1983). Agonists can be classified based on their ability to stimulate a functional response. Full agonists elicit a maximal response, whereas partial agonists result in a submaximal response. Moreover, a partial agonist needs near or complete receptor occupancy to stimulate its maximal response (Hu & El-Fakahany, 1990).

We and others have demonstrated that xanomeline is able to bind to M1-M4 muscarinic receptor subtypes (Bymaster et al., 1997; Christopoulos et al., 1998; Grant & El-Fakahany, 2005)(Chapter 3). However, previous literature has proposed that xanomeline has functional selectivity for the M1 and M4 receptors (Shannon et al., 1994). Xanomeline has been shown to be a full agonist at the M1 receptor utilizing various assays of receptor function (Bymaster et al., 1997; Christopoulos et al., 1998). In contrast, previous research regarding the efficacy of xanomeline at the M3 receptor is contradictory. Bymaster et al. (1997) found that xanomeline was a partial agonist at the M3 receptor; whereas Wood et al. (1999) determined that xanomeline was a full agonist

at this receptor in their model systems. The discrepancy could be due to differences in receptor expression or the expression systems studied.

It is important to compare functional selectivity of xanomeline in the same type of cells that express individual subtypes of muscarinic receptors. This avoids complications caused by factors not related to the type of muscarinic receptor expressed, e.g. type and level of G proteins. Previously, we have shown that there is near equal receptor expression between the M1 and M3 receptors expressed in CHO cells as determined by saturation binding (Chapter 3 Fig. 10). It is also critical to compare the ability of long-term treatment with xanomeline to desensitize various subtypes of muscarinic receptors. Differential rates or extents of receptor desensitization would contribute to agonist selectivity under chronic treatment conditions. Thus, experiments were conducted to test the hypothesis that xanomeline has functional selectivity between the M1 and M3 receptors. We further extended the study to test the hypothesis that xanomeline induces differential long-term effects on subsequent agonist stimulation of M1 and M3 receptors.

Results

Effects of xanomeline pretreatment on M1 and M3 receptor-mediated stimulation of inositol phosphate production

Initial experiments were designed to compare the functional response elicited by xanomeline to that of the M3 full agonist carbachol and the partial agonist pilocarpine (Wang & El-Fakahany, 1993) in untreated cells. The maximal response stimulated by xanomeline was slightly lower than pilocarpine and approximately half that of carbachol, demonstrating that xanomeline displays properties of a partial agonist at the M3 receptor under our specific experimental conditions (Fig. 1 a). However, xanomeline exhibited a significantly ($p < 0.05$) higher potency than either carbachol or pilocarpine (Table 1).

Wash-resistant xanomeline binding resulted in persistent activation of the M3 receptor (Fig. 1 b). Exposure of cells to xanomeline for one minute followed by washing led to a concentration-dependent increase in the hydrolysis of inositol phosphates, with a maximal response similar to that observed when it was continually present with untreated cells, although with a lower potency (Table 1). Interestingly, increasing the preincubation time with xanomeline to one hour prior to washing reduced the maximal response without a change in potency (Table 1).

The functional response of the M1 full agonist carbachol (Wang & El-Fakahany, 1993) was compared to that of xanomeline at the M1 receptor. In contrast to the M3 receptor, xanomeline had a similar maximal response to carbachol, supporting that it is a full agonist at the M1 receptor (Fig. 2 a). However, the potency of xanomeline was significantly ($p < 0.05$) greater compared to carbachol. Results are summarized in Table 1.

Wash-resistant xanomeline was also able to simulate a functional response at the M1 receptor (Fig. 2 b). When cells were exposed to xanomeline for one minute followed by extensive washing there was a slight, but not significant, decrease in the maximal response compared to xanomeline treatment in the functional assay. In contrast, the same treatment resulted in a significant ($p < 0.05$) decrease in potency. When xanomeline pretreatment time was increased to one hour it had no effect on the maximal response, but the potency was only slightly lower than when xanomeline was continually present in the binding assay.

Effects of acute xanomeline exposure on the ability of various muscarinic agonists to stimulate the production of inositol phosphates at the M3 and M1 receptors

Brief pretreatment of cells expressing the M3 receptor with 10 μM xanomeline followed by washing resulted in significant decreases in carbachol potency ($p < 0.05$), with a more marked effect in the case of one hour pretreatment (Fig. 3 a; Table 2). No changes were observed in the maximal response elicited by carbachol following one minute or one hour pretreatment with xanomeline and washing (Fig. 3 a). The concurrent presence of xanomeline and carbachol during the functional assay resulted in a larger reduction in carbachol potency compared to that obtained following either pretreatment condition (Fig. 3 a; Table 2).

Further experiments were conducted to determine the effects of brief xanomeline treatment and washing on the response to partial agonists at the M3 receptor. Pretreatment with 10 μM xanomeline for one minute or one hour followed by washing led to a significant ($p < 0.05$) decrease in pilocarpine potency (by approximately 1 order of magnitude) (Fig. 3 b). This was accompanied by a significant ($p < 0.05$) decrease in pilocarpine maximal response in the one hour-pretreatment group, but not the one-minute group (Fig. 3 b; Table 2). Xanomeline wash-resistant binding also resulted in antagonism of the response to a second addition of xanomeline. This was evidenced by a decrease in xanomeline potency without an alteration in efficacy (Fig. 3 c; Table 2).

Similar experiments were conducted at the M1 receptor to determine the ability of agonists to stimulate a functional response following acute xanomeline pretreatments. We noted that there were slight differences in the binding profiles when assays were performed on cells in suspension versus monolayer (discussed further in Appendix A). Thus, the functional response to carbachol stimulation was determined both in

monolayer and suspension following acute xanomeline pretreatments of cells in monolayer. When cells were treated with 300 nM xanomeline for one minute followed by washing there was no change in carbachol potency for cells assayed in monolayer (Fig. 4 a); however, there was a significant ($p < 0.05$) increase in potency for the same pretreatment condition assayed in suspension (Fig. 4 b). Interestingly, there was a large increase in the basal production of inositol phosphates for cells assayed in suspension, but not for those assayed in monolayer. Neither assay condition resulted in a change in the maximal response to carbachol. When pretreatment time was increased to one hour there was also no change in carbachol maximal response (Fig. 4 c, d). However, there was a large increase in the basal production of inositol phosphates for both assay conditions. This large increase in basal response prevented the potency from being determined for cells assayed in suspension. Taken together, these results demonstrate that the response to carbachol stimulation following acute xanomeline pretreatments is similar, but not identical, for cells assayed in suspension compared to monolayer. Results are summarized in Table 3.

Further experiments were conducted in suspension at the M1 receptor to determine the ability of pilocarpine or xanomeline to stimulate a functional response following acute xanomeline pretreatments. Pretreatment with 300 nM xanomeline for one hour followed by washing did not affect that maximal response to either pilocarpine or xanomeline (Fig. 4 c, d). In contrast, one hour pretreatment with xanomeline resulted in a significant ($p < 0.05$) decrease in xanomeline potency, but not pilocarpine potency compared to their respective controls. Results are summarized in Table 3.

Effects of acute agonist exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the M3 receptor

To determine if the acute effects of xanomeline on agonist stimulation were unique to xanomeline, cells expressing the M3 receptor were exposed to 10 μM pilocarpine for one hour followed by washing and stimulation with a second application of pilocarpine. Under these conditions, there was no change in the potency of pilocarpine and only a slight decrease in the maximal response compared to pilocarpine stimulation in untreated cells (Fig. 5). Results are summarized in Table 2.

Stimulation of inositol phosphates production using exogenous phospholipase C subsequent to short- or long-term treatment with xanomeline at the M3 and M1 receptors

In order to demonstrate that the pool of labeled polyphosphoinositides at either the M3 or M1 receptor was not depleted following short- or long-term xanomeline pretreatments, cells were stimulated with exogenous phospholipase C following the various pretreatments with xanomeline. No differences in the production of inositol phosphates were observed in any of the experimental groups for short-term xanomeline treatments at the M1 (Fig. 6 a) or M3 (Fig. 6 b) receptors or for the long-term xanomeline treatments at the M1 (Fig. 7 a) or M3 (Fig. 7 b) receptors.

Effects of long-term xanomeline exposure on the ability of various muscarinic agonists to stimulate inositol phosphate production at the M3 and M1 receptors

The full agonist carbachol was utilized to determine the long-term effects of xanomeline pretreatment on the sensitivity of the M3 receptor. Pretreatment with 10 μM xanomeline for one minute or one hour followed by washing and waiting for a day

resulted in nearly identical concentration response curves, displaying significant ($p < 0.05$) decreases in the potency of carbachol without significant changes in maximal response compared to control (Fig. 8 a; Table 2). A more marked decrease in carbachol potency (2.4 orders of magnitude) was observed when cells were pretreated with 10 μM xanomeline for 24 hours before washing (Fig. 8 a; Table 2). Again, there was no change in carbachol maximal response.

Lack of demonstrable effects of xanomeline pretreatment on the maximal response elicited by the full agonist carbachol, in spite of the observed large decrease in the number of available cell-surface receptors (Chapter 3 Fig. 19), might be due to the involvement of spare receptors. We therefore tested the effects of xanomeline on the receptor response to partial agonists, as such ligands require full receptor occupancy to elicit maximal receptor activation. Cells were pretreated with 10 μM xanomeline for 24 hours prior to washing, or preincubated with 10 μM xanomeline for either one minute or one hour followed by washing and prolonged waiting in ligand-free media. In all cases, comparable significant ($p < 0.05$) decreases in the maximal response to the partial agonist pilocarpine were observed (Fig. 8 b). These effects were accompanied by a significant ($p < 0.05$) reduction in pilocarpine potency, with a more pronounced effect in case of the 24-hour pretreatment group (Fig. 8 b; Table 2). Of particular note, pretreatment with xanomeline resulted in more marked attenuation of its own stimulatory effects as compared to those of carbachol and pilocarpine (Fig. 8 c). However, xanomeline maximal receptor activation and potency under these conditions could not be quantified since a full concentration response curve could not be attained, due to the concentration limitations presented by using DMSO as a solvent.

As mentioned previously and discussed further in Appendix A, we observed a difference between the results of binding assays conducted in monolayer versus

suspension, so functional assays assessing the ability of 300 nM carbachol to stimulate the production of inositol phosphates subsequent to long-term xanomeline pretreatments were performed both in monolayer and in suspension at the M1 receptor. When cells expressing the M1 receptor were treated with 300 nM xanomeline for one minute or one hour followed by washing and overnight incubation in ligand free media there was no change in the ability of carbachol to stimulate a maximal response for cells assayed in monolayer (Fig. 9 a) or suspension (Fig. 9 b). However, there was a significant ($p < 0.05$) decrease in potency in suspension and a trend in monolayer towards a decrease in the potency following one hour, but not one minute pretreatment in either case. The response to carbachol stimulation following treatment with xanomeline for 24 hours prior to washing was also similar for both monolayer (Fig. 9 a) and suspension (Fig. 9 b) assay groups with a decrease in both potency and maximal stimulation. These results demonstrate that the ability of carbachol to stimulate the production of inositol phosphates following long-term pretreatment with xanomeline is similar between monolayer and suspension assays. Results are summarized in Table 3.

Further experiments were conducted with pilocarpine and xanomeline stimulation in suspension subsequent to long-term xanomeline pretreatments in cells expressing the M1 receptor. In our expression system, pilocarpine and xanomeline are both full agonists at the M1 receptor. When cells were pretreated with 300 nM xanomeline for one hour followed by washing and overnight incubation or treated for 24 hours prior to washing there was a significant ($p < 0.05$) decrease in the maximal stimulation of inositol phosphates in response to pilocarpine, along with a significant ($p < 0.05$) decrease in pilocarpine potency (Fig. 9 c), with a more pronounced effect following 24 hour pretreatment. Similar results were obtained with xanomeline stimulation following 24 hour xanomeline pretreatments. However, xanomeline pretreatment for one hour

followed by washing and waiting 23 hours resulted in only a moderate decrease in the potency of xanomeline and a smaller, yet significant ($p < 0.05$), decrease in maximal stimulation (Fig. 9 d) compared to xanomeline stimulation alone. Results are summarized in Table 3.

Effects of long-term carbachol or pilocarpine exposure on the ability of agonists to stimulate inositol phosphate production at the M3 and M1 receptors

Experiments were conducted to ascertain if the observed delayed effects of wash-resistant xanomeline binding on receptor function at the M3 and M1 receptors (Figs. 8 & 9) were unique to xanomeline or due to general effects elicited by long-term exposure to any muscarinic agonist. A 10 μM concentration of either carbachol or pilocarpine was selected since at this concentration both agonists produce maximal receptor activation and maximal inhibition of [^{3}H]NMS binding following prolonged exposure.

When cells expressing the M3 receptor were pretreated with carbachol or pilocarpine for one hour followed by washing and incubation in control media for 23 hours, no change in efficacy or potency of carbachol in simulating phosphoinositide hydrolysis was observed (Figs. 10 b, c; Table 2). In contrast, pretreatment with carbachol for 24 hours followed by washing resulted in a significant ($p < 0.05$) decrease in the maximal response and potency of a second addition of carbachol (Fig. 10 b; Table 2). A similar long-term treatment with pilocarpine did not alter either concentration-response parameters of carbachol (Fig. 10 c; Table 2). In addition, pretreatment with pilocarpine for one hour followed by washing and waiting did not modify the concentration-response relationship of pilocarpine. However, pilocarpine treatment for 24 hours prior to washing followed by a second addition of pilocarpine demonstrated

significant ($p < 0.05$) decreased potency as well as a marked decrease in efficacy (Fig. 10 d), similar to what was observed with carbachol pretreatment followed by carbachol stimulation (Fig. 10 b).

Similar experiments utilizing carbachol stimulation following carbachol pretreatment were conducted in cells expressing the M1 receptor. When cells were pretreated with 10 μM carbachol for one hour followed by overnight incubation in ligand free media there was no difference in the potency or efficacy following carbachol stimulation compared to control (Fig. 10 a). When cells were pretreated with carbachol for 24 hours prior to washing there was no change in efficacy following a second addition of carbachol to stimulate the production of inositol phosphates compared to carbachol stimulation alone (Fig. 10 a). In contrast, the same treatment resulted in a substantial decrease in the potency of carbachol (Fig. 10 a). Results are summarized in Table 3.

Further experiments were performed to determine the effects of 24 hour carbachol pretreatment on the ability of xanomeline to stimulate a response at either the M3 or M1 receptor (Fig. 11). Pretreatment of cells expressing the M3 receptor for 24 hours 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 μM) (Fig. 11 b; Table 2). Pretreatment of cells expressing the M1 receptor for 24 hours with carbachol prior to xanomeline stimulation also resulted in minimal production of inositol phosphates (Fig. 11 a; Table 3). However, the response occurred with lower concentrations of xanomeline than needed at the M3 receptor.

Discussion

The functional consequences of xanomeline treatment were studied by determining the accumulation of inositol phosphates (Figs. 1 & 2). Xanomeline was found to be a potent partial agonist at the M3 muscarinic receptor (Fig. 1) and a potent full agonist at the M1 muscarinic receptor (Fig. 2). This is in agreement with previous studies (Bymaster et al., 1997; Christopoulos et al., 1998). However, Wood et al. (1999) showed that xanomeline acted as a full agonist at M3 receptors in their expression system. This discrepancy may be due to different receptor expression levels or variation in the functional assays employed to assess receptor sensitivity. The wash-resistant component of xanomeline binding established after exposure to the drug for one minute was able to stimulate a concentration-dependent functional response at both the M3 and M1 receptor. The maximal response at the M3 receptor following one-minute xanomeline exposure and washing was similar to that observed when xanomeline was present during the functional assay, albeit with a lower potency. In contrast, there was a slight decrease in efficacy accompanied by a decrease in potency following one minute xanomeline treatment and washing compared to the presence of xanomeline during the functional assay at the M1 receptor. When pretreatment with xanomeline was increased to one hour followed by washing it resulted in a slightly lower efficacy compared to xanomeline continuously present in the assay at the M3 and M1 receptors. This time-dependent reduction in receptor sensitivity might be due to xanomeline-induced desensitization of the receptor. It has been demonstrated that an agonist can cause desensitization without changing receptor expression (Maloteaux & Hermans, 1994).

The notion that desensitization was occurring without changing cell-surface receptor density was tested further by examining the effects of xanomeline treatment on the ability of other agonists to stimulate the production of inositol phosphates (Figs. 4 &

5). When xanomeline was present for one minute or one hour followed by washing and immediate use it decreased the potency of carbachol without reduction of its maximal response at both the M3 and M1 receptors. These results are in agreement with saturation binding experiments in which there was marked reduction in the affinity of the radioligand for either receptor in the absence of change in receptor density. However, at the M3 receptor, pretreatment with xanomeline for one hour resulted in wash-resistant attenuation of not only the potency of the partial agonist pilocarpine, but also its maximal response. This might be due to the higher susceptibility of partial agonists to changes in receptor sensitivity (Hu et al., 1991). Alternatively, these results could be explained by differential allosteric modulation by xanomeline of the physical interaction of different agonists with the receptor (Lanzafame et al., 2006; May et al., 2005). In contrast, pretreatment with xanomeline for one hour followed by washing did not have an effect on potency or efficacy of pilocarpine stimulation at the M1 receptor. This may be because pilocarpine is a full agonist at the M1 receptor. At either the M3 or M1 receptor, pretreatment with xanomeline followed by subsequent stimulation with itself resulted in a reduction of the potency with no change in efficacy. Interestingly, xanomeline and pilocarpine are both partial agonists at the M3 receptor; however, the effects of xanomeline pretreatment prior to their stimulation are slightly different, further suggesting allosteric modulation of the receptor by xanomeline.

The long-term effects of xanomeline pretreatment are more marked than the acute effects. When cells expressing the M3 receptor were treated with xanomeline for one minute or one hour followed by washing and prolonged waiting there was a decrease in the potency of both carbachol and pilocarpine, albeit the shift in potency was less pronounced than that observed following acute xanomeline pretreatments (Fig. 8). This corresponds well to the observed reversal of the effects of xanomeline on [^{3}H]NMS

affinity following prolonged incubation of pretreated and washed cells in control media. In contrast, there was a greater decrease in the potency of both carbachol and pilocarpine following brief xanomeline treatment prior to washing and waiting at the M1 receptor, compared to acute xanomeline pretreatments (Fig. 9). This discrepancy could be a result of differential modulation of the receptor between subtypes. In contrast, wash-resistant xanomeline binding followed by waiting at both the M3 and M1 receptors resulted in additional delayed reduction in the maximal functional response elicited by carbachol and pilocarpine that is likely due to reduction in cell-surface receptor density detected in radioligand binding experiments. Pretreatment with xanomeline for 24 hours prior to washing resulted in a more pronounced effect on the potency of carbachol and pilocarpine compared to brief xanomeline pretreatment followed by washing and waiting at both subtypes. This might be due to the larger decrease in receptor potency detected in radioligand binding studies under the latter treatment conditions. Something else might be happening at the M1 receptor since the decrease in radioligand affinity is not greater for 24 hours. In contrast, when cells expressing the M3 receptor were briefly treated with xanomeline followed by washing and further incubation or treated for 24 hours prior to washing it markedly reduced the ability of xanomeline to stimulate a subsequent response. This reduction was to a greater extent than was seen with other agonists, suggesting the possibility of differential allosteric modulation of the receptor by xanomeline. However, at the M1 receptor, the reduction in the ability of xanomeline to stimulate a response following pretreatment with itself was not as attenuated. This further suggests a slight difference in xanomeline regulation between the M1 and M3 receptors.

The observed delayed effects of brief 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 (Fig. 10). When cells expressing the M3 receptor were pretreated with pilocarpine for one hour followed by washing and waiting 23 hours or for 24 hours prior to washing, no changes in carbachol-induced receptor stimulation were observed. Similarly, pretreatment with carbachol for one hour followed by washing and waiting 23 hours did not alter the ability of carbachol to elicit a response at either the M3 or M1 receptors. However, when cells expressing either the M3 or M1 receptor were treated for 24 hours 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. 10). 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 (Hu et al., 1991; Yang et al., 1993). Pretreatment of cells expressing the M3 receptor with pilocarpine produced changes in its ability to subsequently activate the receptor similar to those described above for carbachol. Contradictory reports of receptor regulation by pilocarpine exist. Previous research has shown that prolonged exposure to pilocarpine results in a decrease in cell-surface receptor density (Shifrin & Klein, 1980). In contrast, another report suggested that pilocarpine has little effect on the actual number of receptors, as the apparent down-regulation was due to pilocarpine being released when cells were lysed prior to the binding assay (Milligan & Strange, 1983). However, in our paradigm 24-hour exposure of cells expressing the M3 receptor to pilocarpine prior to washing resulted in a decrease in cell-surface receptor density (Fig. 10). 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 (Hu & El-Fakahany, 1990). Additionally, 24-hour pretreatment with carbachol resulted in a larger reduction in the ability of xanomeline to stimulate a functional response at either the M3 or M1 receptors compared to stimulation by other agonists. Thus, these results further demonstrate that xanomeline interacts in a unique manner with muscarinic receptors.

In summary, we have demonstrated that xanomeline is a potent partial agonist at the M3 receptor and a potent full agonist at the M1 receptor. Additionally, wash-resistant xanomeline can stimulate a functional response at both receptor subtypes. Acute pretreatment with xanomeline is able to modulate the response to subsequent stimulation by various agonists. Similarly, prolonged exposure to xanomeline results in antagonism of the response to subsequent stimulation with agonists. This antagonism was slightly more pronounced at the M3 receptor compared to the M1 receptor. These effects are unique to xanomeline rather than being a general property of conventional agonists. These results are in agreement with the slight differences observed between the M1 and M3 receptor in radioligand binding studies. Furthermore, these results demonstrate that xanomeline is able to modulate receptor function through binding at an allosteric site on the receptor.

Figure 1

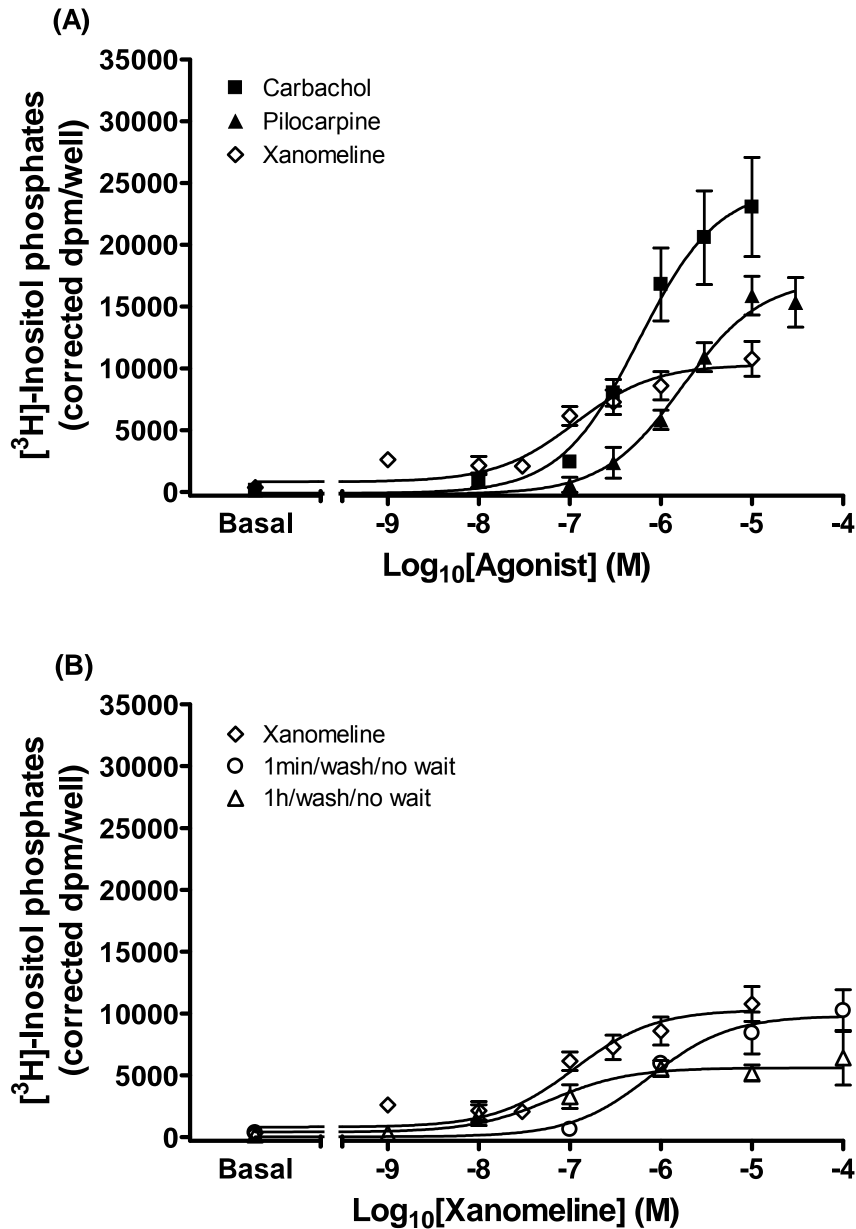


Figure 1. hM3 receptor activation by xanomeline reversible and wash-resistant binding. A) Cells were incubated at 37°C for 1 hour with increasing concentrations of carbachol (closed squares), pilocarpine (closed triangles) or xanomeline (open diamonds) in the presence of 10 mM lithium chloride. B) Cells were pretreated with increasing concentrations of xanomeline for 1 minute (open circles) or 1 hour (open diamonds) followed by washing and determination of the accumulation of inositol phosphates by wash-resistant xanomeline. In the third group (open diamonds) xanomeline was only present during determination of inositol phosphates accumulation. Values represent the means ± S.E. of 3-5 experiments conducted in triplicate.

Figure 2

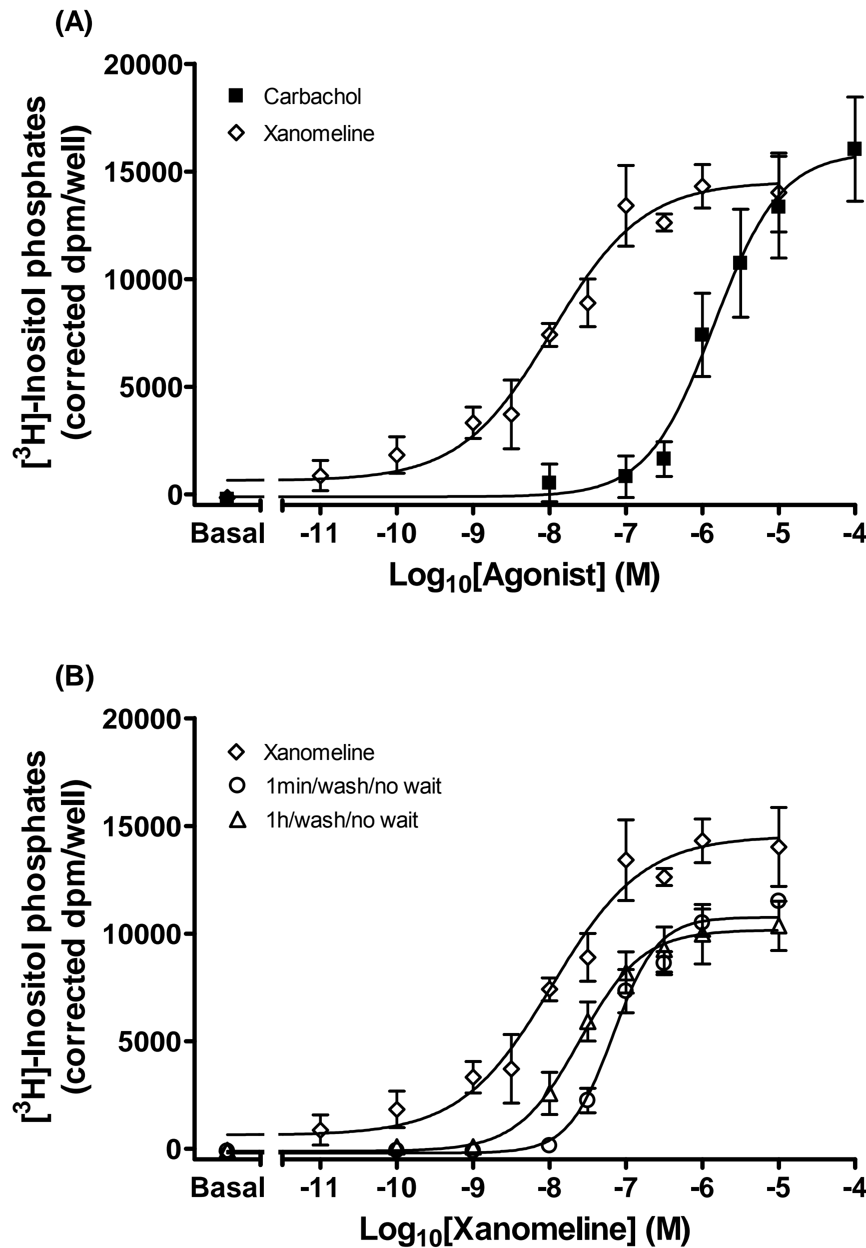


Figure 2. hM1 receptor activation by xanomeline reversible and wash-resistant binding. A) Cells were incubated at 37°C for 1 hour with increasing concentrations of carbachol (closed squares) or xanomeline (open diamonds) in the presence of 10 mM lithium chloride. B) Cells were pretreated with increasing concentrations of xanomeline for 1 minute (open circles) or 1 hour (open diamonds) followed by washing and determination of the accumulation of inositol phosphates by wash-resistant xanomeline. In the third group (open diamonds) xanomeline was only present during determination of inositol phosphates accumulation. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate. Data provided by Marianne Grant.

Figure 3

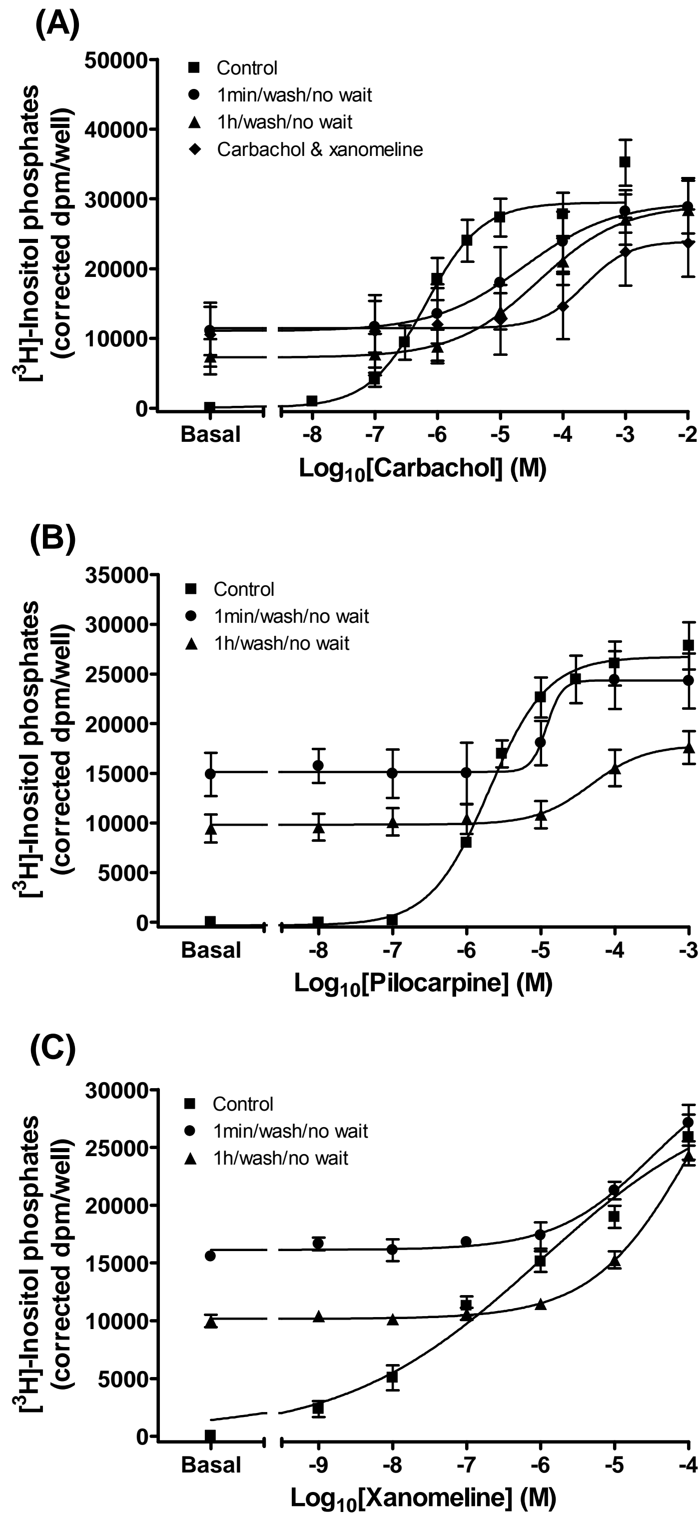


Figure 3. Effects of acute xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM3 receptor. Cells were pretreated with 10 μM xanomeline for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing and immediate use. Subsequently, cells were treated with increasing concentrations of A) carbachol, B) pilocarpine, or C) xanomeline and the accumulation of inositol phosphates was determined over a 1-hour incubation period at 37°C in the presence of 10 mM lithium chloride for untreated cells (closed squares) and those pretreated with xanomeline. A) An additional group of cells was simulated simultaneously with increasing concentrations of carbachol and 10 μM xanomeline (closed diamonds). Values represent the means ± S.E. of 3-7 experiments conducted in triplicate.

Figure 4

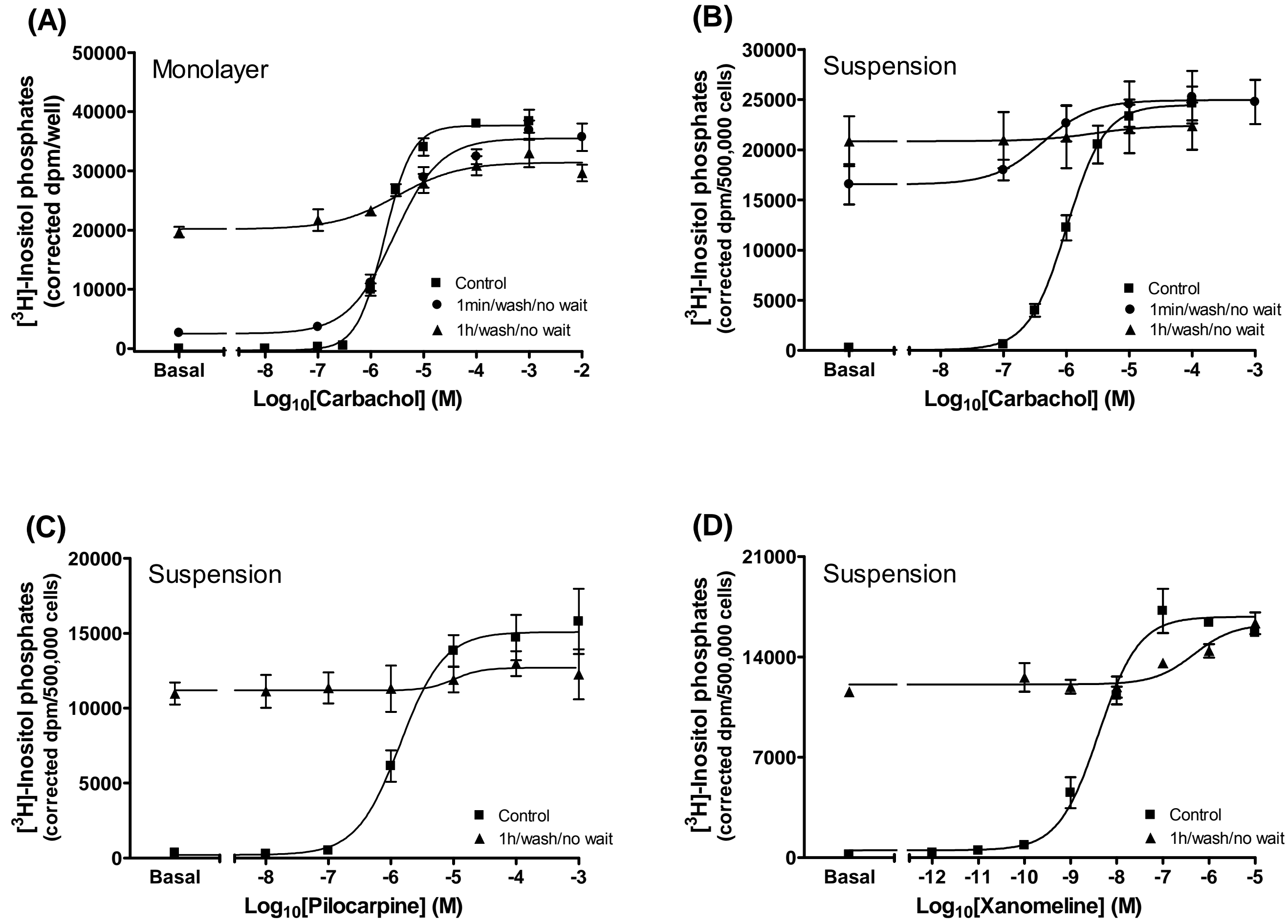


Figure 4. Effects of acute xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM1 receptor. Cells were pretreated with 300 nM xanomeline for 1 minute (closed circles) or 1 hour (closed triangles) followed by washing and immediate use. A) Untreated (closed squares) or xanomeline pretreated cells were incubated with increasing concentrations of carbachol in monolayer for 1 hour at 37ºC in the presence of 10 mM lithium chloride to determine inositol phosphate production. Alternatively, cells in suspension were treated with increasing concentrations of B) carbachol, C) pilocarpine, or D) xanomeline and the accumulation of inositol phosphates was determined over a 1-hour incubation period at 37°C in the presence of 10 mM lithium chloride for untreated cells (closed squares) and those pretreated with xanomeline. Values represent the means ± S.E. of 3-9 experiments conducted in triplicate. Data from (C) and (D) provided by Kayla De Lorme.

Figure 5

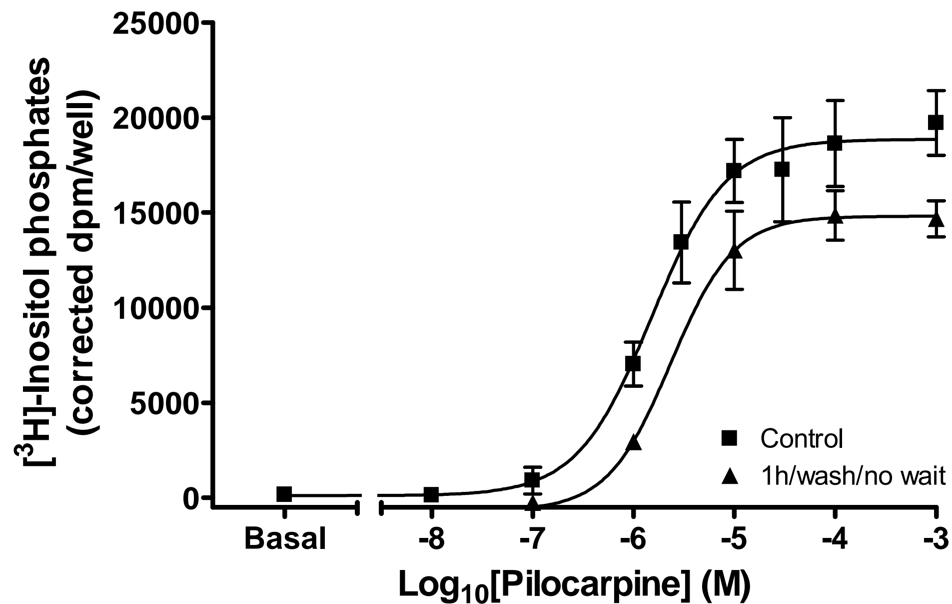


Figure 5. Effects of acute pilocarpine on the ability of pilocarpine to subsequently stimulate the production of inositol phosphates at the hM3 receptor. Cells expressing the M3 receptor were pretreated for 1 hour with 10 μM pilocarpine followed by washing (closed triangles). Subsequently, untreated (closed squares) or pretreated cells were stimulated with increasing concentrations of pilocarpine for 1 hour at 37ºC in the presence of 10 mM lithium chloride to determine the production of inositol phosphates. Values represent the mean ± S.E. of 3 experiments conducted in triplicate.

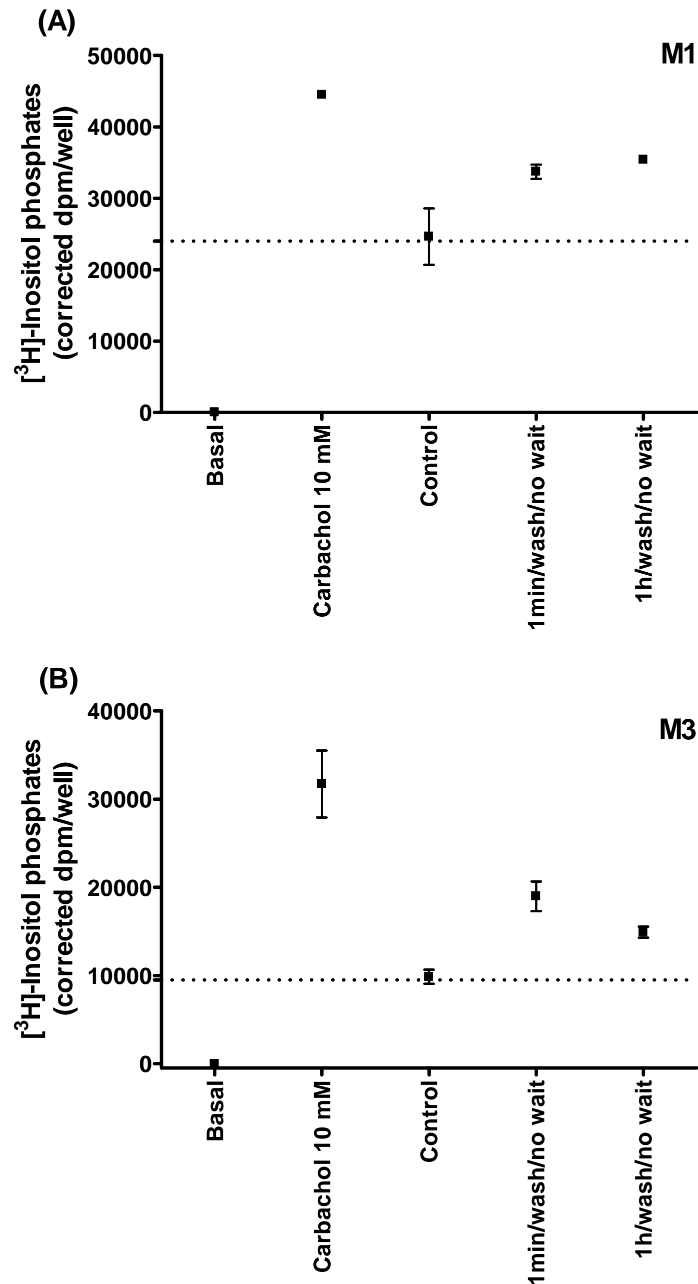


Figure 6. Stimulation of inositol phosphate production by exogenous phospholipase C following brief xanomeline pretreatments in cells expressing M1 and hM3 receptors. Cells were pretreated with xanomeline for 1 minute or 1 hour followed by washing. Subsequently, untreated or pretreated cells were stimulated with 2.5 U of phospholipase C for 1 hour at 37ºC in the presence of 10 mM lithium chloride and the level of inositol phosphate production was determined. As a reference untreated cells were unstimulated or stimulated with 10 µM carbachol instead of phospholipase C. A) M1 receptor and B) M3 receptor. Values represent the mean of 2 experiments conducted in triplicate.

Figure 7

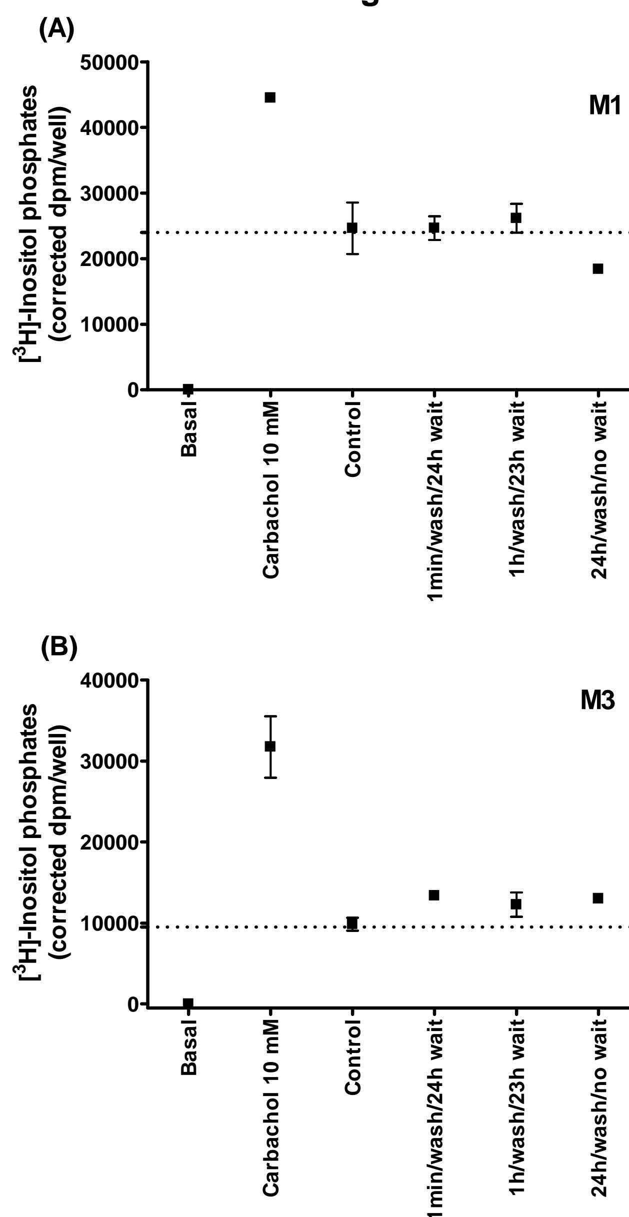


Figure 7. Stimulation of inositol phosphate production by exogenous phospholipase C following long-term xanomeline pretreatments in cells expressing hM1 and hM3 receptors. Cells were pretreated with xanomeline for 1 minute or 1 hour followed by washing and incubation in control media for 24 or 23 hours, respectively. Additional cells were treated with xanomeline for 24 hours prior to washing. Subsequently, untreated or pretreated cells were stimulated with 2.5 U of phospholipase C for 1 hour at 37ºC in the presence of 10 mM lithium chloride and the production of inositol phosphates was determined. As a reference untreated cells were unstimulated or stimulated with 10 µM carbachol instead of phospholipase C. A) M1 receptor and B) M3 receptor. Values represent the mean of 2 experiments conducted in triplicate.

Figure 8

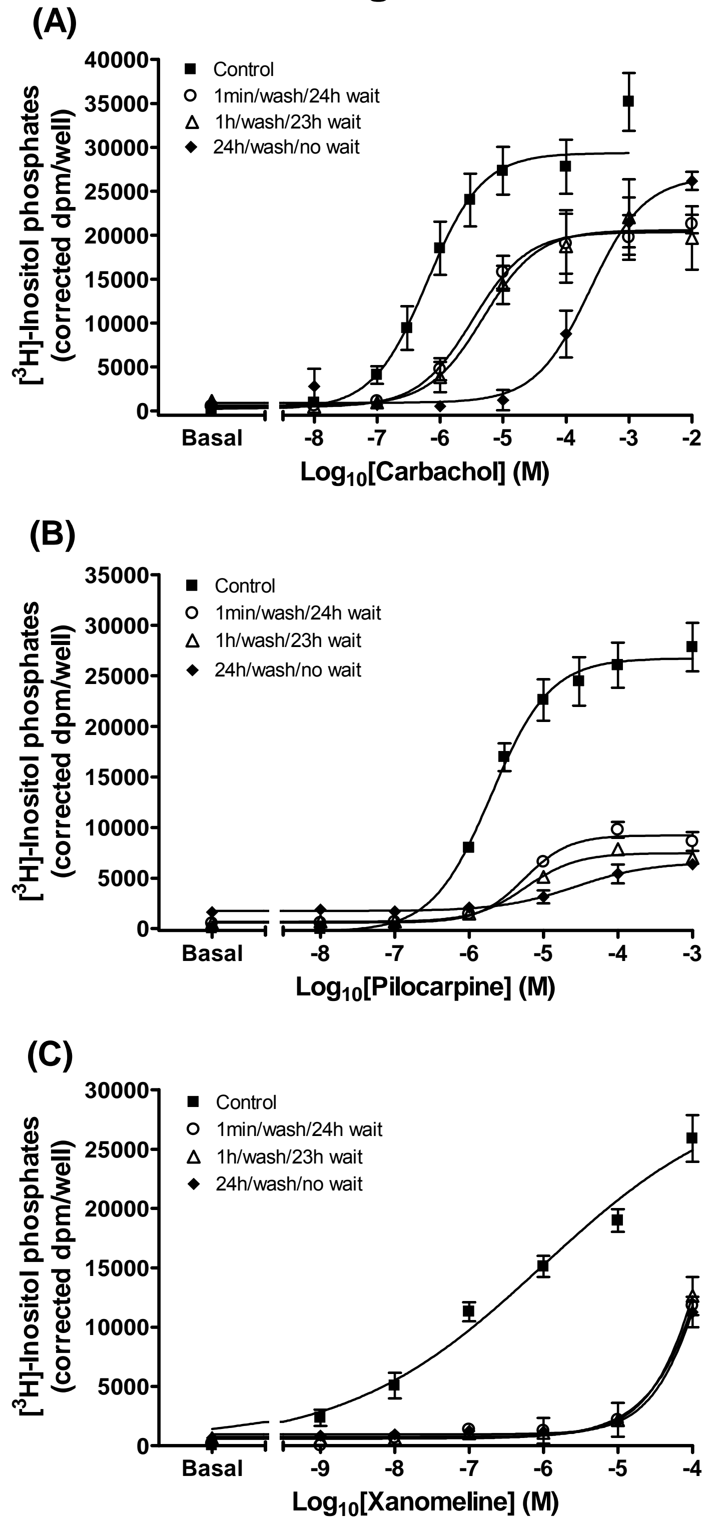


Figure 8. Effects of prolonged xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM3 receptor. Cells were pretreated with 10 μM xanomeline for 1 minute (open circles) or 1 hour (open triangles) followed by washing and waiting 24 or 23 hours, respectively. An additional group of cells was treated with xanomeline for 24 hours (closed diamonds) prior to washing. Subsequently, cells were treated with increasing concentrations of A) carbachol, B) pilocarpine, or C) xanomeline and the accumulation of inositol phosphates was determined over a 1-hour incubation period at 37°C in the presence of 10 mM lithium chloride for untreated cells (closed squares) or those pretreated with xanomeline. Values represent the means ± S.E. of 3-7 experiments conducted in triplicate.

Figure 9

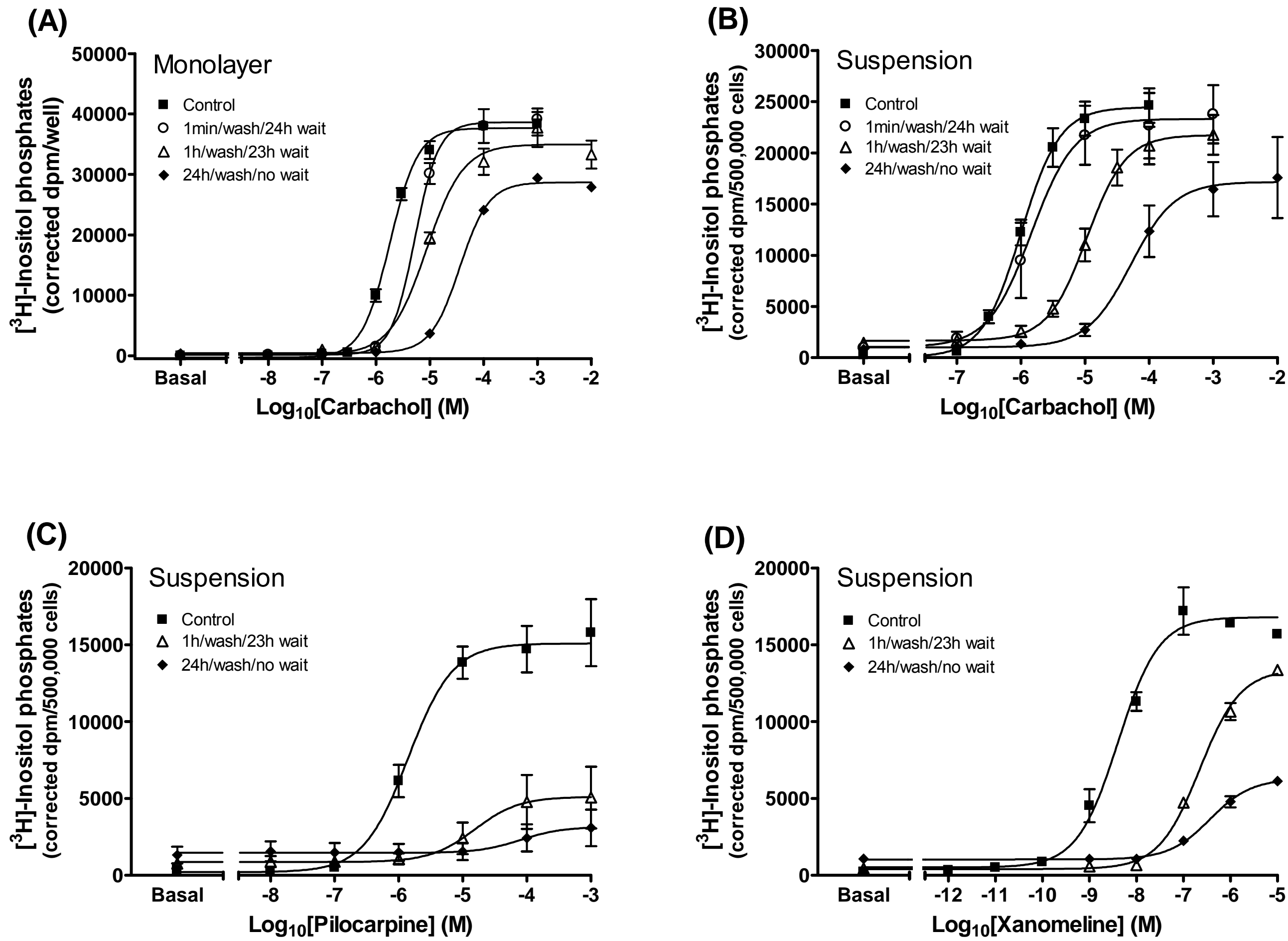


Figure 9. Effects of prolonged xanomeline exposure on the ability of muscarinic agonists to stimulate the production of inositol phosphates at the hM1 receptor. Cells were pretreated with 300 nM xanomeline for 1 minute (open circles) or 1 hour (open triangles) followed by washing and waiting 24 or 23 hours, respectively. An additional group of cells was treated with xanomeline for 24 hours (closed diamonds) prior to washing. A) Untreated (closed squares) or xanomeline pretreated cells were incubated with increasing concentrations of carbachol in monolayer for 1 hour at 37ºC in the presence of 10 mM lithium chloride to determine inositol phosphate production. Alternatively, cells were treated with increasing concentrations of B) carbachol, C) pilocarpine, or D) xanomeline in suspension and the accumulation of inositol phosphates was determined over a 1-hour incubation period at 37°C in the presence of 10 mM lithium chloride for untreated cells (closed squares) or those pretreated with xanomeline. Values represent the means ± S.E. of 2-9 experiments conducted in triplicate. Data from (C) and (D) provided by Kayla De Lorme.

Figure 10

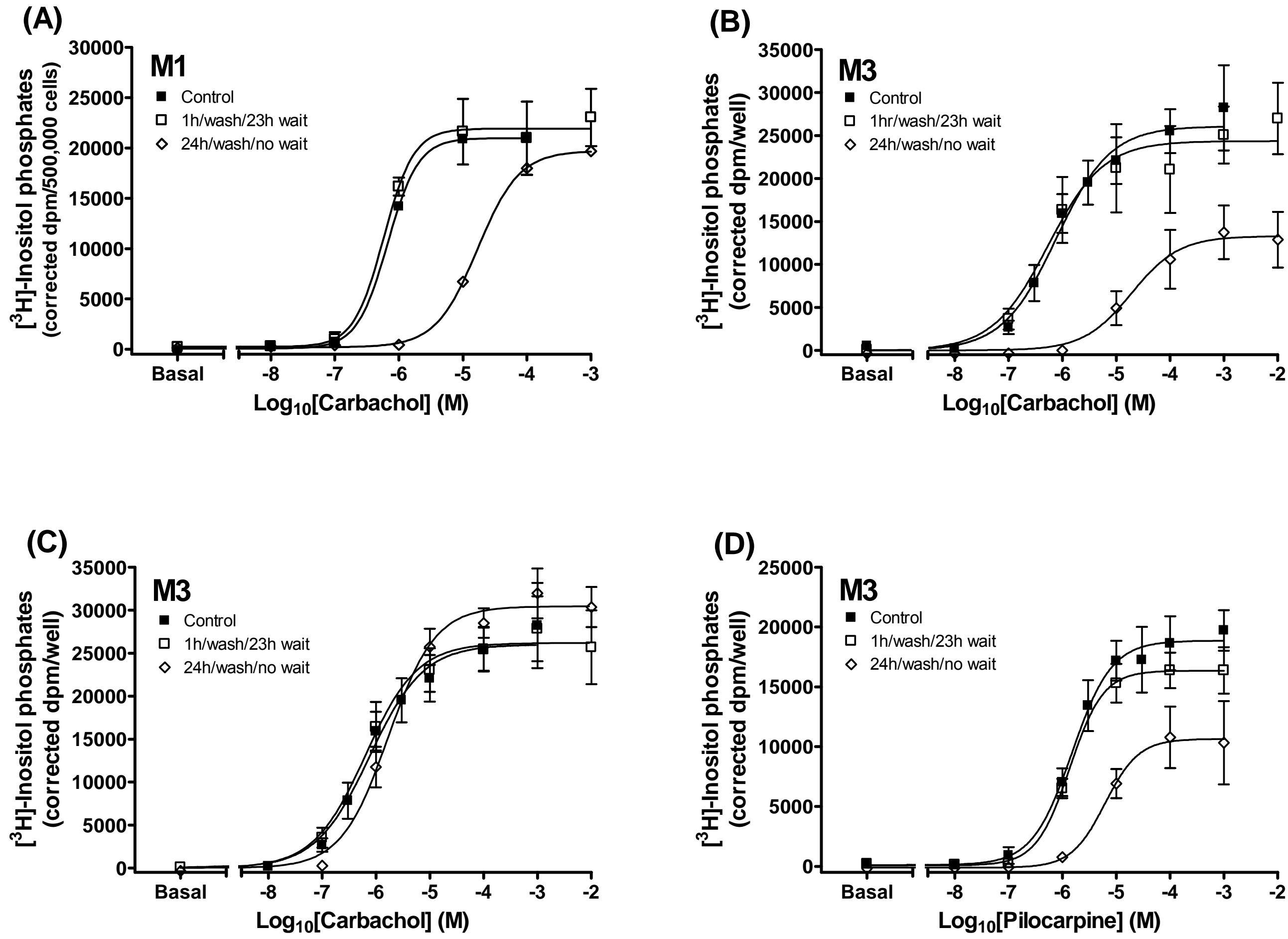


Figure 10. Effects of long-term agonist exposure on the ability of various muscarinic agonists to stimulate inositol phosphates production at the hM1 and hM3 receptors. A) Cells expressing the M1 receptor were treated with 10 μM carbachol for 1 hour followed by washing and waiting 23 hours in control media (open squares) or treated for 24 hours with carbachol prior to washing (open diamonds). Subsequently, cells were stimulated in suspension with increasing concentrations of carbachol for 1 hour at 37ºC in the presence of 10 mM lithium chloride to determine inositol phosphate production. B) Cells expressing the M3 receptor were pretreated with 10 μM carbachol as in (A). Subsequently, cells were stimulated in monolayer with increasing concentrations of carbachol and the accumulation of inositol phosphates was determined in the presence of 10 mM LiCl over a 1 hour incubation period at 37ºC for untreated cells (closed squares) and those pretreated with carbachol. C) Cells expressing the M3 receptor were treated with 10 μM pilocarpine similar to (B) then stimulated with carbachol. D) Cells expressing the M3 receptor were treated with 10 μM pilocarpine as in (B) then stimulated with pilocarpine. Values represent the means ± S.E. of 2-6 experiments conducted in triplicate.

Figure 11

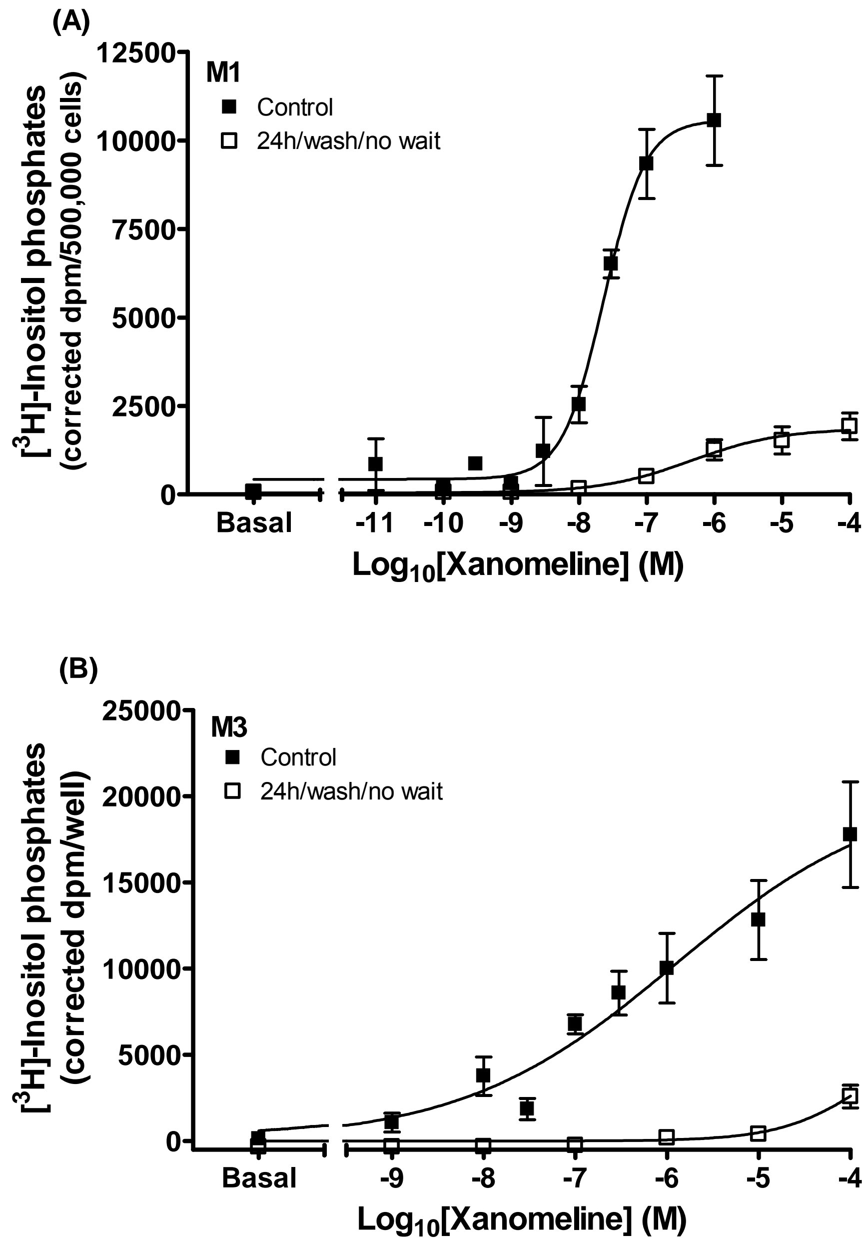


Figure 11. Effects of long-term exposure to carbachol on the ability of xanomeline to stimulate inositol phosphate production at hM1 and hM3 receptors. Cells were pretreated with 10 µM carbachol for 24 hours prior to washing (open squares). Subsequently, untreated (closed squares) or pretreated cells were stimulated with increasing concentrations of xanomeline for 1 hour at 37ºC in the presence of 10 mM lithium chloride and the production of inositol phosphates was determined. A) Experiments were conducted at the M1 receptor in suspension. B) Experiments were conducted at the M3 receptor in monolayer. Values represent the means of ± S.E. of 3 experiments conducted in triplicate. Data in (A) provided by Marianne Grant.

Table 1. Effects of agonist treatment on PI hydrolysis in CHO cells expressing hM1 and hM3 receptor subtypes. Cells were incubated at 37°C for 1 hour with increasing concentrations of carbachol (10 nM – 100 μM), pilocarpine (100 nM – 4.5 μM) or xanomeline (1 nM – 10 μM) and the levels of inositol phosphate production were determined in the presence of 10 mM lithium chloride. Additional groups of cells were treated with xanomeline (1 nM – 100 μM) for 1 minute or 1 hour followed by washing and use in the functional assay. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	M1		**M3**	
	pEC_{50}[a]	**E_{max}**[b]	**pEC_{50}**	**E_{max}**
Agonist stimulation				
Carbachol	5.8 ± 0.24	15800 ± 2600	6.3 ± 0.04	23300 ± 3900
Pilocarpine			5.8 ± 0.08	16400 ± 2100
Xanomeline	8.0 ± 0.11[†]	14700 ± 1400	6.9 ± 0.07[†]	11100 ± 1300*
Xanomeline pretreatment				
1 min/washout/no wait	7.1 ± 0.08[$]	11100 ± 350	6.1 ± 0.10	9800 ± 2000
1 h/washout/no wait	7.6 ± 0.11	10200 ± 1200	7.1 ± 0.52	6200 ± 1300

[a] Negative logarithm of the midpoint (potency) parameter.
[b] Maximal response. Values are expressed as dpm/well after correcting for standard recovery.
No statistical difference in E_{max} was detected at the M1 receptor between xanomeline and carbachol.
[$] ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in EC_{50} where indicated between xanomeline 1min/wash/no wait or 1h/wash/no wait compared with xanomeline at the M1 receptor.
[†] ANOVA followed by Tukey's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} between xanomeline compared with carbachol or pilocarpine at the M1 or M3 receptor.
* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in E_{max} between xanomeline compared with carbachol at the M3 receptor.
No statistical differences in E_{max} or pEC_{50} were found between the xanomeline-pretreated groups compared to xanomeline at the M3 receptor.

Table 2. Effects of carbachol, pilocarpine or xanomeline stimulation following short- and long-term agonist pretreatment and washout on inositol phosphates production in CHO cells expressing hM3 receptors. Cells were pretreated with 10 µM agonists for 1 minute or 1 hour followed by washing and immediate use in the functional assay or subsequent to incubation for 24 or 23 hours, respectively, in the absence of free agonist. Additional groups of cells were treated with 10 µM agonist for 24 hours prior to washing. In the functional assay, cells were stimulated with increasing concentrations of carbachol, pilocarpine or xanomeline for 1 hour at 37°C in the presence of 10 mM lithium chloride. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	Carbachol Stimulation		**Pilocarpine Stimulation**		**Xanomeline Stimulation**	
	pEC_{50}[a]	**E_{max}[b]**	**pEC_{50}**	**E_{max}**	**pEC_{50}**	**E_{max}**
Xanomeline						
Control (untreated)	6.2 ± 0.09	28300 ± 3100	5.7 ± 0.004	26800 ± 2300	6.2 ± 0.14	28100 ± 3000
1 min/washout/no wait	4.7 ± 0.19*	30700 ± 4100	4.9 ± 0.05†	24500 ± 2800	4.5 ± 0.10‡	32200 ± 2800
1 h/washout/no wait	4.2 ± 0.06*	28900 ± 5100	4.3 ± 0.05†	17900 ± 1500†	4.4 ± 0.15‡	32200 ± 2800
Xanomeline & carbachol – co-addition	3.7 ± 0.11*	24000 ± 4800				
1 min/washout/24h wait	5.5 ± 0.14*	20400 ± 2000	5.3 ± 0.06†	9200 ± 880†	ND	ND
1 h/washout/23h wait	5.1 ± 0.10*	25600 ± 2700	5.2 ± 0.05†	7000 ± 600†	ND	ND
24 h/washout/no wait	3.6 ± 0.20*	26800 ± 1000	4.0 ± 0.04†	6600 ± 500†	ND	ND
Carbachol						
Control (untreated)	6.1 ± 0.11	25400 ± 2400			5.7 ± 0.35	21300 ± 3300
1 h/washout/23 h wait	6.0 ± 0.43	25600 ± 3300				
24 h/washout/no wait	4.7 ± 0.27$	13200 ± 3200$			ND	ND

Table 2 cont.

	Carbachol Stimulation		Pilocarpine Stimulation		Xanomeline Stimulation	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Pilocarpine						
Control (untreated)	6.1 ± 0.11	25400 ± 2400	5.8 ± 0.04	19000 ± 1900		
1 h/washout/23 h wait	6.2 ± 0.08	26300 ± 3400	5.9 ± 0.03	16400 ± 1600		
24 h/washout/no wait	5.8 ± 0.11	30500 ± 2300	5.3 ± 0.11[#]	10800 ± 3200		

[a] Negative logarithm of the midpoint (potency) parameter.
[b] Maximal response. Values are expressed as dpm/well after correcting for standard recovery.
ND – parameter value was not determined
* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with carbachol stimulation compared to carbachol stimulation in untreated cells.
† ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with pilocarpine stimulation compared to pilocarpine stimulation in untreated cells.
‡ ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with xanomeline stimulation compared to xanomeline stimulation in untreated cells.
$ ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between carbachol-pretreated groups with carbachol stimulation compared to carbachol stimulation in untreated cells.
# ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between pilocarpine-pretreated groups with pilocarpine compared to pilocarpine stimulation in untreated cells.
No significant difference was detected between pilocarpine-pretreated groups with carbachol stimulation compared to carbachol stimulation in untreated cells.

Table 3. Effects of carbachol, pilocarpine or xanomeline stimulation following short- and long-term agonist pretreatment and washout on inositol phosphates production in CHO cells expressing hM1 receptors. Cells were pretreated with 300 nM xanomeline or 10 μM carbachol for 1 minute or 1 hour followed by washing and immediate use in the functional assay or subsequent to incubation for 24 or 23 hours, respectively, in the absence of free agonist. Additional groups of cells were treated with 300 nM xanomeline or 10 μM carbachol for 24 hours prior to washing. In the functional assay cells, were stimulated with increasing concentrations of carbachol, pilocarpine or xanomeline for 1 hour at 37°C in the presence of 10 mM lithium chloride. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	Carbachol Stimulation		Pilocarpine Stimulation		Xanomeline Stimulation	
Pretreatment	pEC_{50} [a]	E_{max} [b]	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Xanomeline – suspension [c]						
Control (untreated)	6.0 ± 0.06	24500 ± 1800	5.8 ± 0.12	13900 ± 1700	7.9 ± 0.40	18200 ± 1200
1 min/washout/no wait	6.4 ± 0.18*	25100 ± 2200				
1 h/washout/no wait	ND	21000 ± 2100	5.6 ± 0.75	12800 ± 1000	6.4 ± 0.55$	17900 ± 1200
1 min/washout/24 h wait	5.9 ± 0.10	23300 ± 2900				
1 h/washout/23 h wait	5.0 ± 0.06*	21100 ± 2400	4.3 ± .029†	6300 ± 1800†	6.6 ± 0.03	11600 ± 2200$
24 h/washout/no wait	4.3 ± 0.11*	14700 ± 2800*	3.9 ± 0.29†	3800 ± 960†	6.3 ± 0.09$	5100 ± 1200$
Xanomeline – monolayer [d]						
Control (untreated)	5.7	37700				
1 min/washout/no wait	5.6	35500				
1 h/washout/no wait	5.5	31500				
1 min/washout/24 h wait	5.3	38600				
1 h/washout/23 h wait	5.1	35000				
24 h/washout/no wait	4.4	28700				

Table 3 cont.

	Carbachol Stimulation		Pilocarpine Stimulation		Xanomeline Stimulation	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Carbachol						
Control (untreated)	6.2	21000			7.7 ± 0.11	10900 ± 1100
1 h/washout/23 h wait	6.2 ± 0.15	21900 ± 3300				
24 h/washout/no wait	4.8	19700			6.6 ± 0.01[‡]	1600 ± 370[‡]

[a] Negative logarithm of the midpoint (potency) parameter.
[b] Maximal response. Values are expressed as dpm/well after correcting for standard recovery.
[c] Pretreatments were conducted in monolayer and the functional assay was conducted in suspension.
[d] Pretreatments and the functional assay were conducted on cells in monolayer. The values listed are for a single assay conducted in triplicate.
ND – parameter not determined
* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with carbachol stimulation compared to carbachol stimulation in untreated cells.
[†] ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with pilocarpine stimulation compared to pilocarpine stimulation in untreated cells.
[$] ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} where indicated between xanomeline-pretreated groups with xanomeline stimulation compared to xanomeline stimulation in untreated cells.
[‡] Unpaired t-test detected a significant difference ($p < 0.05$) in pEC_{50} and E_{max} between carbachol 24h/wash/no wait with xanomeline stimulation compared to xanomeline stimulation in untreated cells.

CHAPTER 5: Receptor activation is required for the development of the long-term effects of xanomeline at the M1 and M3 muscarinic receptors

Introduction

Binding of conventional ligands to muscarinic receptors occurs at the orthosteric site of the receptor. This binding results in activation of down-stream signaling cascades through conformational changes in transmembrane domains 3, 5, 6 and 7 (Eglen, 2005). The M1 and M3 receptors are coupled to $G_{q/11}$ G proteins that lead to the activation of the phospholipase C signaling pathway (Volpicelli & Levey, 2004). Prolonged activation of the receptor through the orthosteric binding site can lead to changes in receptor expression and a decrease in the ability of subsequent agonist treatment to stimulate a functional response (Hu et al., 1991; Maloteaux & Hermans, 1994). Additionally, binding of a drug to an allosteric site on a receptor may lead to changes in the conformation of the receptor's orthosteric domain that can result in receptor activation (Christopoulos & Kenakin, 2002; Wess, 2005). Examples of such allosteric muscarinic receptor agonists include AC-42 (Spalding et al., 2002) and WIN51708 (Lanzafame et al., 2006).

Xanomeline has been shown to bind in a wash-resistant manner at the M1 receptor (Christopoulos et al., 1998; Jakubik et al., 2002). Further results demonstrated that binding occurs in this manner even when the primary binding site is blocked (Jakubik et al., 2002). When the orthosteric site is blocked acutely with atropine, stimulation of the receptor is inhibited; however, when atropine is washed out xanomeline is again able to stimulate a functional response (Christopoulos et al., 1998). Xanomeline has been shown to bind in an allosteric manner; however, it is unclear if activation through the orthosteric site is necessary for the development of the long-term consequences of this binding.

Thus, the aim of the study was to determine if the orthosteric site is necessary for the long-term consequences of xanomeline binding at the M1 and M3 receptors. To determine this, we used a variety of protocols to block receptor function in CHO cells

expressing either M1 or M3 muscarinic receptors. Our data demonstrate that receptor activation through the orthosteric site is necessary for the long-term consequence of xanomeline on receptor binding and functional properties. However, xanomeline binding in the absence of function is still able to regulate the affinity of the receptor for other ligands.

Results

Effects of atropine on long-term xanomeline-induced inhibition of [^{3}H]NMS binding at the M1 and M3 receptors

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 receptor-saturating concentration of atropine (10 μM), a muscarinic receptor antagonist, was added either concurrently with 10 μM xanomeline pretreatment or during the waiting period following xanomeline washout. [^{3}H]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 at the M3 receptor. The simultaneous presence of atropine during incubation of cells with xanomeline for one hour followed by washing and waiting 23 hours in control medium did not influence the significant ($p < 0.05$) decrease in [^{3}H]NMS maximal binding induced by xanomeline pretreatment alone (Fig. 1 a, b; Table 1). 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. 1 c; Table 1). In contrast, either protocol of atropine treatment abolished the long-term effects of xanomeline on radioligand affinity. All treatment groups had similar protein content.

When parallel experiments were conducted at the M1 receptor, similar results were seen to those obtained at the M3 receptor. When atropine (10 μM) and xanomeline (3 μM) were simultaneously present for one hour followed by washing and waiting, the significant ($p < 0.05$) decrease in maximal binding was not affected compared to xanomeline pretreatment alone (Fig. 2 a, b). In contrast, when cells were treated with atropine for 23 hours subsequent to one hour xanomeline pretreatment the long-term changes in maximal binding were no longer evident (Fig. 2 c). Additionally, in both cases atropine treatment prevented the change in radioligand binding affinity observed with xanomeline treatment alone (Fig. 2 b, c). Results are summarized in Table 1.

Effects of atropine on xanomeline-induced long-term alterations in receptor sensitivity at the M1 and M3 receptors

Similar to the previously described [^{3}H]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 μM), either concurrently with 10 μM xanomeline pretreatment for one hour or during the 23 hour waiting period following xanomeline pretreatment and washing on the ability of increasing concentrations of carbachol to stimulate the production of inositol phosphates in cells expressing the M3 receptor. In contrast to results obtained from binding experiments, simultaneous presence of atropine with xanomeline for one hour followed by washing and waiting for 23 hours in control medium abolished the effects of xanomeline on carbachol potency (Fig. 3 a, b; Table 1). The presence of atropine during the 23 hour incubation following xanomeline treatment and washout also eliminated xanomeline-induced changes in

carbachol potency, although a decrease in efficacy was observed compared to control (Fig. 3 a, c; Table 1).

Functional experiments were also employed using atropine (10 μM) to block the orthosteric site of the M1 receptor to determine the effects of blocking receptor function on the ability of increasing concentrations of carbachol to stimulate inositol phosphate production subsequent to long-term exposure to xanomeline (300 nM). When cells were treated concurrently with atropine and xanomeline for one hour followed by washing and waiting 23 hours, the effect on carbachol potency was no longer evident compared to xanomeline pretreatment alone (Fig. 4 a, b), similar to the observed effects at the M3 receptor. However, the basal level of receptor stimulation was elevated at the M1 receptor under these treatment conditions, which was not observed with xanomeline treatment alone (Fig. 4 b). The reason for the increase in basal stimulation is unclear. Similar to the results obtained with saturation binding, the presence of atropine during the 23 hour waiting period subsequent to xanomeline pretreatment abolished the changes observed with xanomeline treatment alone (Fig. 4 c). Additionally, no change in maximal stimulation was evident with either atropine pretreatment condition. Results are summarized in Table 1.

Effects of blocking receptor function, using a non-functional mutant, on inhibition of [^{3}H]NMS and saturation binding parameters at the M1 receptor

Experiments were designed to further test the necessity of receptor activation on the long-term effects of xanomeline binding at the M1 receptor using a non-functional receptor mutant (rM1[123]) expressed in CHO cells. In this case, the mutant receptor was cloned from rat so additional parallel experiments were conducted with the rat wild-type receptor (rM1). Lack of a functional response of the receptor mutant was confirmed

under our experimental conditions (Fig. 5 a). Exposure of wild-type rM1 receptors to carbachol or xanomeline indicted that xanomeline is a partial agonist in stimulating inositol phosphate production. This is in contrast to the hM1 receptor where xanomeline and carbachol had similar efficacies (Fig. 5 a; Chapter 4 Fig. 2 a). However, at either wild-type receptor, human or rat, xanomeline was significantly ($p < 0.05$) more potent than carbachol (Fig. 5 a; Chapter 4 Fig. 2 a). Subsequently, inhibition of [^{3}H]NMS binding experiments were conducted to compare the ability of xanomeline to regulate the rM1 and rM1^{123} mutant receptors.

When cells expressing either the rM1 or rM1^{123} receptors were exposed to increasing concentrations of xanomeline in the binding assay, the potencies and maximal inhibition of [^{3}H]NMS binding were similar between receptors (Fig. 5 b, c; Table 2). Similarly, when cells were exposed to xanomeline for one hour followed by washing and used immediately in the binding assay the potencies and maximal inhibition of [^{3}H]NMS binding were nearly identical between the receptors. However, the potency decreased by approximately 1.3 – 1.6 orders of magnitude compared to the former treatment. In contrast, when cells were exposed to xanomeline for one hour followed by washing and waiting 23 hours a biphasic binding curve was observed at the rM1 receptor, but a monophasic binding curve was observed at the rM1^{123} mutant receptor (Fig. 5 b, c). In this case the potency at the low-potency site for the rM1 receptor was similar to the potency at the rM1^{123} mutant receptor. Similar results were observed in the binding isotherms following 24 hour xanomeline treatment prior to washing, with a biphasic binding curve observed at the rM1 and a monophasic binding curve at the rM1^{123} mutant receptor. Interestingly, the potency for 24 hour xanomeline treatment followed by washing was indistinguishable from the potency of the acute xanomeline treatment at the rM1^{123} mutant receptor (Fig. 5 c). In the case of long-term exposure to

xanomeline, inhibition of $[^3H]$NMS binding was complete for all groups except xanomeline treatment for one hour followed by washing and further incubation in control media for 23 hours. In that case the binding curve was still decreasing at the highest concentrations of xanomeline tested, but the vehicle that xanomeline was dissolved in (DMSO) prohibited testing at higher concentrations. Results are summarized in Table 2.

Additionally, saturation binding experiments were conducted at the rM1 wild-type and $rM1^{123}$ mutant receptors to further determine the importance of receptor function on cell-surface receptor density and radioligand binding affinity. In both cases, when cells were exposed to xanomeline (3 μM) for one hour followed by washing and immediate use there was no change in cell-surface receptor density, but a decrease in radioligand affinity compared to their respective controls (Fig. 5 d, e). When rM1 expressing cells were treated with xanomeline for one hour followed by washing and waiting 23 hours there was a significant ($p < 0.05$) decrease in cell-surface receptor density with no change in radioligand affinity compared to control (Fig. 5 d). In contrast, the decrease in cell-surface receptor density compared to control was absent in cells expressing the $rM1^{123}$ mutant receptor (Fig. 5 e). Similarly, when cells expressing the wild-type rM1 receptor were exposed to xanomeline for 24 hours prior to washing there was a decrease in cell-surface receptor density along with a significant ($p < 0.05$) decrease in radioligand affinity (Fig. 5 d). In contrast, the decrease in cell-surface receptor density was absent and the decrease in radioligand affinity was less pronounced in cells expressing the $rM1^{123}$ mutant receptor (Fig. 5 e). Results are summarized in Table 2.

Due to the fact that xanomeline was dissolved in dimethyl sulfoxide (DMSO), control experiments were designed to ensure that DMSO did not contribute to the observed effects of xanomeline. Cells were treated with DMSO concentrations corresponding to those in contact with cells during the various treatment modalities with

xanomeline in the inhibition of [^{3}H]NMS binding assay. DMSO did not affect radioligand binding at the highest concentrations used to prepare xanomeline dilutions (1%; Fig. 6 a, b) in either case. Additionally, protein content was determined following DMSO pretreatments for the various conditions. Once again, there was no change in protein content for any of the treatment groups (Fig. 6 c, d).

As previously mentioned, there was a decrease in protein expression following xanomeline treatments at the M1 receptor, thus protein analysis was conducted using the various xanomeline pretreatments previously described. In this case there was no difference in protein content for any of the aforementioned xanomeline pretreatments (Fig. 6 e, f).

Effects of blocking receptor function using siRNA targeted against G_q and G_{11} G proteins at the M3 receptor

Experiments were conducted to further examine the role of receptor activation on the long-term effects of xanomeline treatments on [^{3}H]NMS saturation binding parameters at the M3 receptor. It is known that the M3 receptor exerts its functional response via activation of the $G_{q/11}$ class of G proteins (Volpicelli & Levey, 2004). Thus, we utilized siRNA technology to block expression of these G proteins. To this effect, cells were transfected with 50 nM siRNA targeting G_q and G_{11} G proteins in an effort to block receptor function. Western blot analysis confirmed that transfection with siRNA targeting $G_{q/11}$ G proteins resulted in a nearly 100 percent decrease in the detectable expression levels of $G_{q/11}$ class of G proteins (Fig. 7 a). In addition, loss of receptor function following transfection was assessed by determining agonist-mediated production of inositol phosphates using two concentrations of carbachol (1 μM and 100 μM) or xanomeline (100 nM and 10 μM). The response to carbachol was decreased by

62 and 27 percent, respectively, and the response to xanomeline was reduced by 78 and 82 percent, respectively (Fig. 7 b). The ability of agonist 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 once again 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 one hour, wash and immediate use xanomeline pretreatment as well as pretreatment with xanomeline for 24 hours prior to washing (Fig. 7 c; Table 3). Additionally, a trend toward a decrease in maximal binding resulted following one hour xanomeline pretreatment followed by washing and waiting 23 hours or for 24 hour xanomeline treatment followed by washing and no waiting (Fig. 7 c; Table 3). 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 (Chapter 3 Fig. 19; Chapter 3 Table 2). This is similar to previous work by Thompson & Fisher (1990) in which there was a greater loss of receptors in cells that were pretreated with carbachol in monolayer versus those in 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 which had a significant ($p <$ 0.05) decrease in radioligand affinity compared to control. There was no change in maximal binding for any of the siRNA-treated groups compared to control (Fig. 7 d; Table 3). Protein content remained the same across the various pretreatment groups.

Differential role of wash-resistant binding versus persistent receptor activation in xanomeline long-term effects on saturation binding at the M3 receptor

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 (Jakubik et al., 2004). Thus, experiments were conducted at the M3 receptor to determine the ability of xanomeline analogs to bind in a reversible and wash-resistant manner. The analogs studied were hexyl (xanomeline), pentyl (one carbon shorter), propyl (three carbons shorter) and ethyl (four carbons shorter). All analogs tested were able to decrease [^{3}H]NMS binding in a concentration dependent manner with complete inhibition when included in the binding assay (Fig. 8 a). The rank order of potencies was pentyl > hexyl ≈ propyl > ethyl. When cells were treated with the various analogs for one hour followed by washing and used immediately there was a concentration dependent decrease in [^{3}H]NMS binding for all except the ethyl analog (Fig. 8 b). In this case the rank order of potencies was hexyl ≈ pentyl > propyl. Additionally, the maximal inhibition of [^{3}H]NMS binding was greater for the hexyl and pentyl analogs compared to the propyl analog (Table 5). In the assay of receptor function, the propyl analog produced 45% of the PI response to xanomeline; however, it did not display any wash-resistant receptor activation (Fig. 8 c). 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 μM for one hour followed by washing and overnight incubation in control media, there was only a minute decrease in cell-surface receptor density in conjunction with a slight increase in radioligand affinity (Fig. 8 d). 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 along with a significant ($p < 0.05$) decrease in the affinity of [^{3}H]NMS (Fig. 8 d). Results are summarized in Table 5.

Discussion

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's primary domain by xanomeline's active head group (Christopoulos et al., 1999; Christopoulos et al., 1998; Jakubik et al., 2002). 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. 1 & Fig. 2). 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 at either the M1 or M3 receptor, suggesting that activation of the receptor during the initial treatment period is not necessary in order for xanomeline to induce long-term changes in either case. 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 (Christopoulos et al., 1998; De Lorme et al., 2006; De Lorme et al., 2007). 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 at both the M1 and M3 receptor. Together, these results indicate that receptor activation is necessary to produce the long-term consequences of xanomeline wash-resistant binding at both the M1 and M3 receptor.

Functional experiments were conducted to determine the effects of suppressing receptor activation on the delayed actions of xanomeline at M1 and M3 receptors (Fig. 3 & Fig. 4). 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 PI hydrolysis observed following xanomeline treatment alone at both the M1 and M3 receptors. 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. However, at the M1 receptor, the basal level of inositol phosphate production was elevated following simultaneous pretreatment with atropine and xanomeline prior to washing and waiting. The presence of atropine during the waiting period following xanomeline pretreatment prevented the change in carbachol potency observed with xanomeline treatment alone at both subtypes. In comparison, the presence of atropine during the 23 hour incubation following acute xanomeline treatment resulted in a decrease in carbachol efficacy compared to atropine treatment alone at the M3 receptor, which was not evident at the M1 receptor. This is in contrast to the observed antagonism by atropine of xanomeline-induced changes in [^{3}H]NMS saturation binding at the M3 receptor. The discrepancies between the results obtained in saturation binding compared to the functional response assays 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 (Nelson et al., 2006; Thor et al., 2008). For example, previous research found that continuous treatment with atropine can lead to an increase in receptor expression (Lee & Wolfe, 1989; May et al., 2005). Thus, the effects of atropine itself may be concealing the functional effects of long-term xanomeline exposure.

A mutant rM1 receptor was used to further explore the role of receptor activation on the development of the long-term effects of xanomeline at the rM1 receptor. Functional assays were performed to confirm that the mutation resulted in a complete loss of the ability of agonists to stimulate the production of inositol phosphates at the $rM1^{123}$ mutant receptor (Fig. 5 a). When cells were exposed acutely to xanomeline it resulted in wash-resistant binding in a concentration dependent manner at both the wild-type and mutant rM1 receptors (Fig. 5 b, c). Interestingly, overnight incubation in control media results in a biphasic binding curve at the wild-type receptor, but a monophasic binding curve for the mutant receptor. Similar results were observed following xanomeline treatment for 24 hours prior to washing. Taken together, these results suggest that receptor function is necessary to develop the prolonged effects of xanomeline binding. The similarity in potency between cells expressing $rM1^{123}$ receptors treated with xanomeline for one hour followed by washing and immediate use and those treated with xanomeline for 24 hours prior to washing suggests that persistently bound xanomeline may interfere with [^{3}H]NMS binding in the absence of receptor function. To further support this conclusion saturation binding experiments were conducted employing the various xanomeline pretreatments. Under these conditions, prolonged treatment with xanomeline resulted in a decrease in cell-surface receptor density for wild-type, but not $rM1^{123}$ mutant receptors (Fig. 5 d, e). Interestingly, 24 hour treatment with xanomeline prior to washing resulted in a decrease in radioligand affinity for both the wild-type and mutant receptors, however, it was more pronounced at the wild-type receptor. Thus, these results demonstrate that receptor function is necessary for the long-term effects of xanomeline on cell-surface receptor density, but receptor function is not solely responsible for the long-term effects on radioligand affinity.

To further explore the importance of receptor activation in the long-term effects of xanomeline at the M3 receptor, receptor function was obliterated using siRNA targeted against the G_q and G_{11} G proteins (Fig. 7). Functional assays and western blots confirmed that receptor response and $G_{q/11}$ 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. In contrast, treatment with xanomeline for 24 hours followed by washing resulted in a decrease in radioligand affinity at both siRNA-treated and untreated M3 receptors, further supporting the notion that xanomeline binding can initiate changes in radioligand affinity in the absence of function. This further suggests allosteric regulation of the primary binding site by wash-resistant xanomeline binding.

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 (Jakubik et al., 2004). 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 [^{3}H]NMS saturation binding paradigms (Fig. 8), pretreatment with the propyl analog for one hour followed by washing and prolonged incubation resulted in a very small decrease in cell-surface receptor density 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 hours prior to washing, whereby a marked decrease in cell-surface receptor density was observed. Taken together, these results suggest that wash-resistant agonist binding in the absence of persistent receptor activation is not the major contributor to the long-term effects of xanomeline binding.

In summary, xanomeline has previously been shown to bind in both a reversible and wash-resistant manner at the M1 and M3 receptors. This binding followed by prolonged exposure results in long-term changes in the binding and functional properties at both receptor subtypes. These results demonstrate that receptor activation is necessary for the majority of the long-term receptor regulation by xanomeline. However, wash-resistant xanomeline binding is able to allosterically modulate the primary binding site, even in the absence of receptor function.

Figure 1

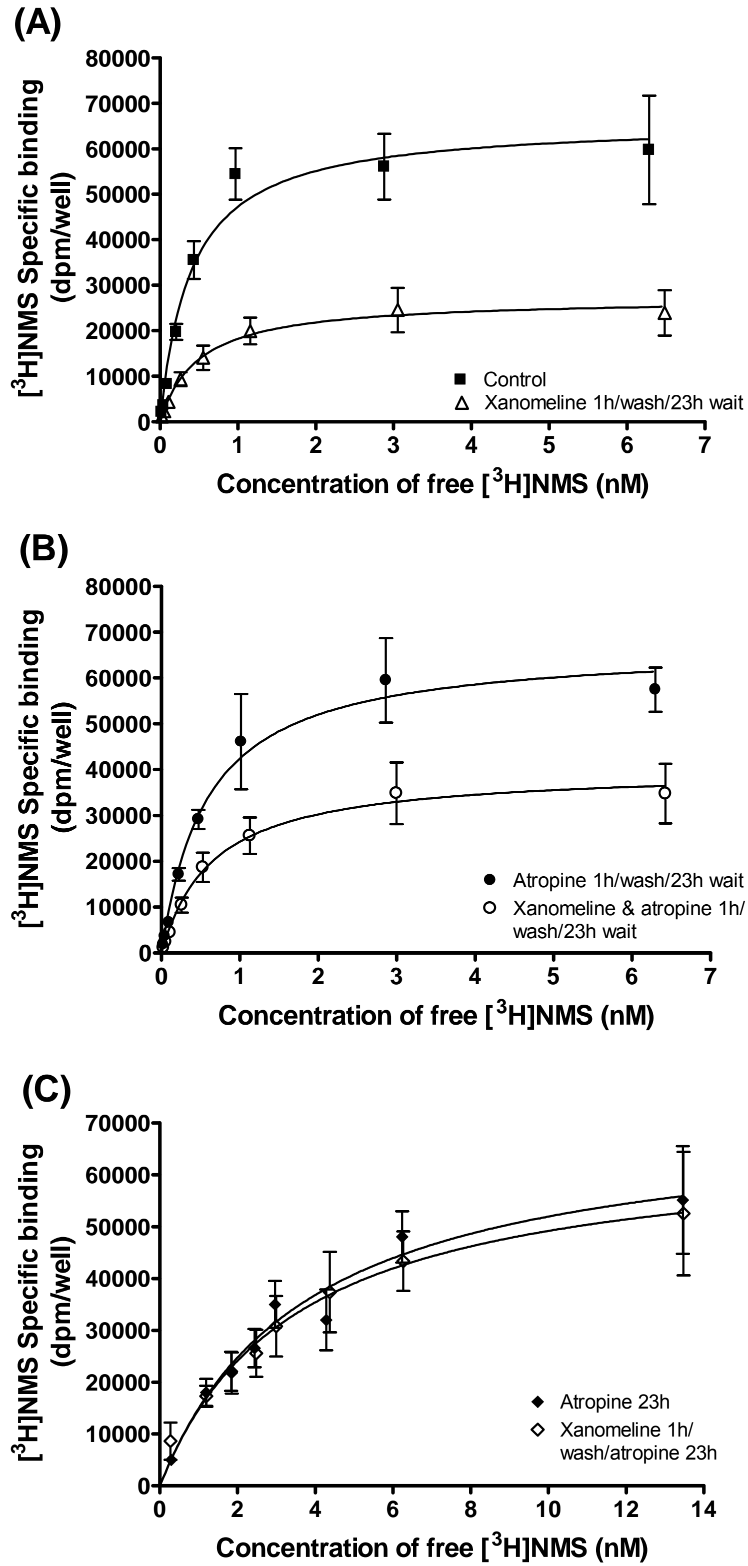


Figure 1. Effects of atropine on long-term xanomeline-induced inhibition of [^{3}H]NMS binding in CHO cells expressing hM3 receptors. A) Cells were either untreated (closed squares) or treated with 10 μM xanomeline for 1 hour followed by washing and waiting for 23 hours (open triangles). B) Cells were treated with 10 μM atropine for 1 hour followed by washing and waiting 23 hours in control medium (closed circles) or treated simultaneously with atropine and xanomeline for 1 hour followed by washing and waiting 23 hours in control medium (open circles). C) Cells were treated with atropine for 23 hours prior to washing (closed diamonds) or with xanomeline for 1 hour followed by washing and incubation with atropine for 23 hours prior to washing (open diamonds). Saturation binding assays were conducted with increasing concentrations of [^{3}H]NMS (0.01 – 14 nM) for 1 hour at 37°C. Values represent the means ± S.E. of 4 experiments conducted in duplicate.

Figure 2

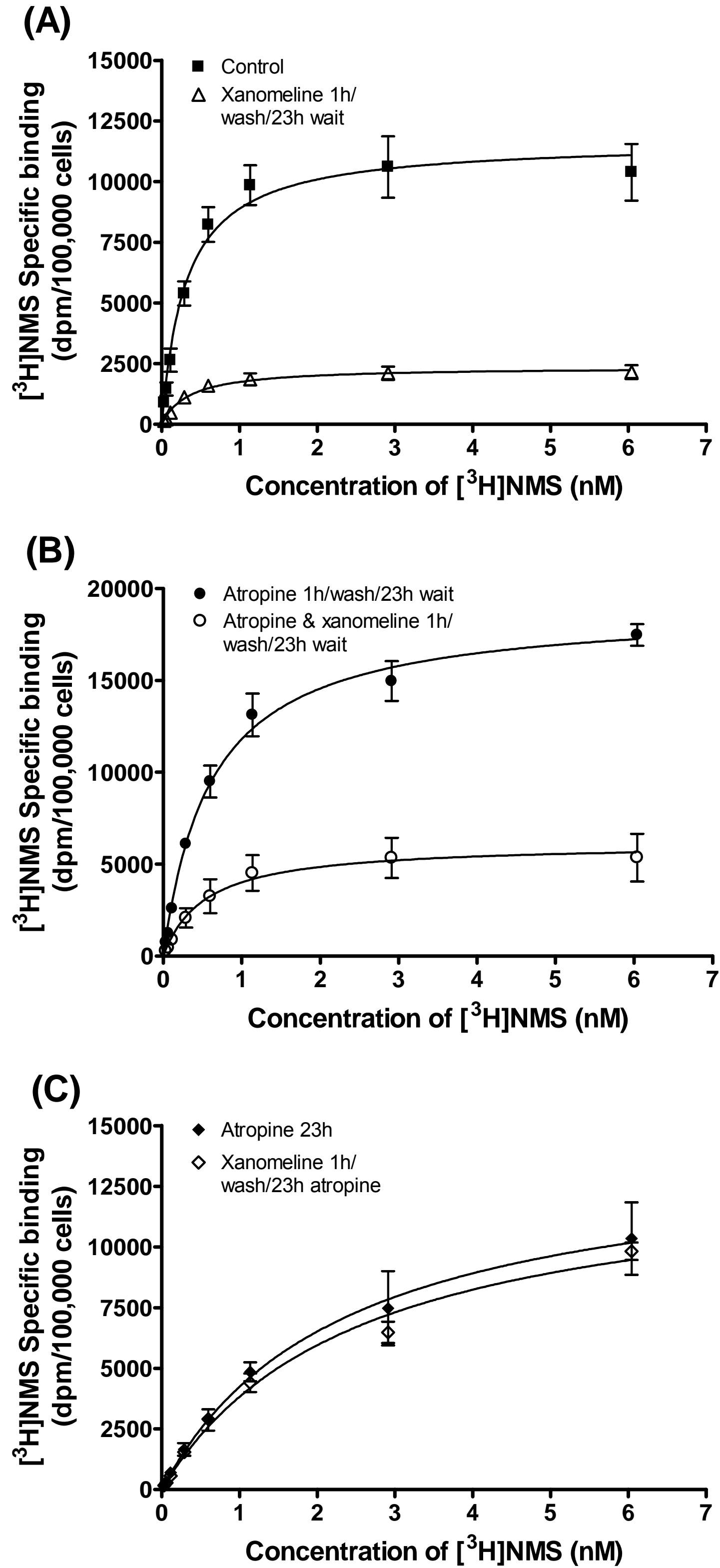


Figure 2. Effects of atropine on long-term xanomeline-induced inhibition of [^{3}H]NMS binding in CHO cells expressing hM1 receptors. A) Cells were either untreated (closed squares) or treated with 3 μM xanomeline for 1 hour followed by washing and waiting for 23 hours (open triangles). B) Cells were treated with 10 μM atropine for 1 hour followed by washing and waiting 23 hours in control medium (closed circles) or treated simultaneously with atropine and xanomeline for 1 hour followed by washing and waiting 23 hours in control medium (open circles). C) Cells were treated with atropine for 23 hours prior to washing (closed diamonds) or with xanomeline for 1 hour followed by washing and incubation with atropine for 23 hours prior to washing (open diamonds). Saturation binding assays were conducted in suspension with increasing concentrations of [^{3}H]NMS (0.02 – 4.5 nM) for 1 hour at 37°C. Values represent the means ± S.E. of 3 experiments conducted in triplicate. Data provided by Marianne Grant.

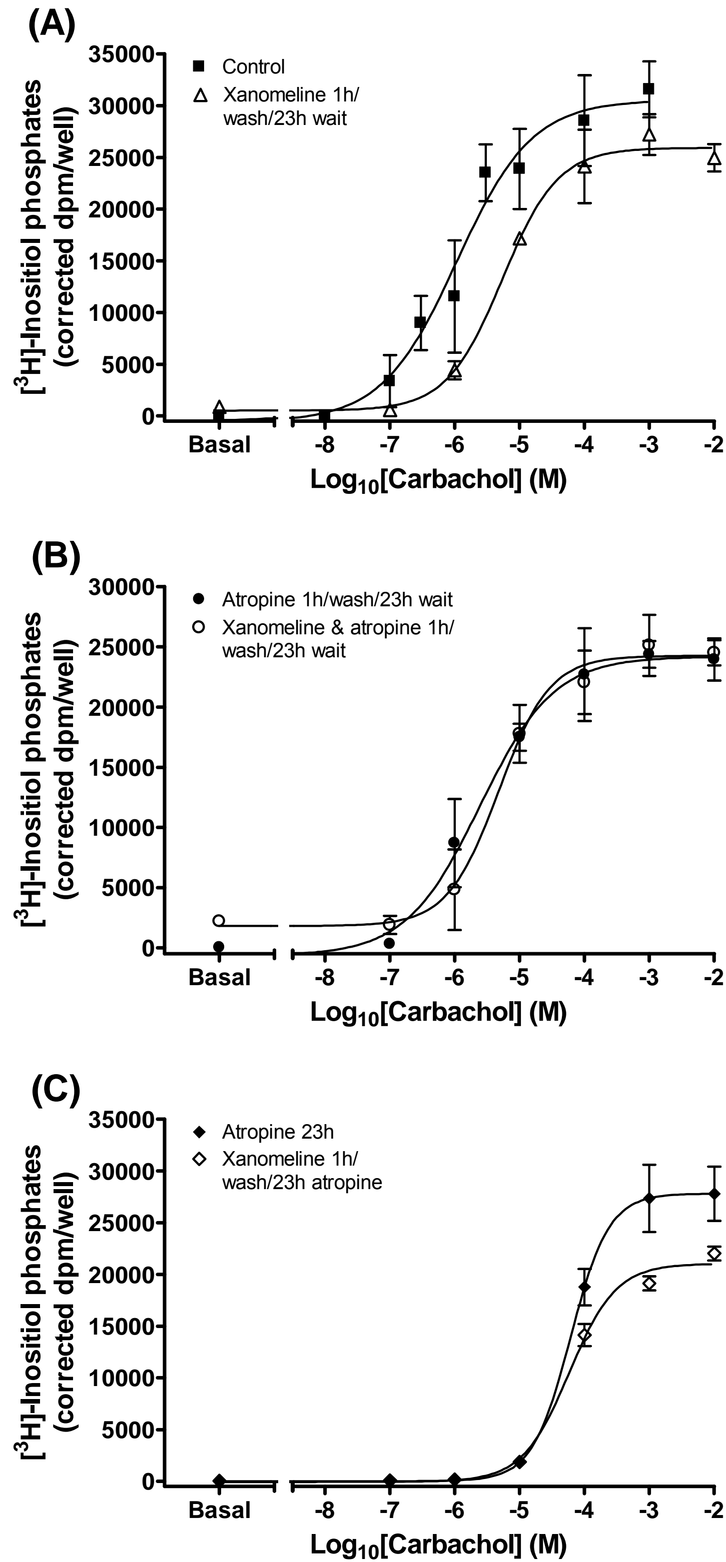


Figure 3
(A)
Control
Xanomeline 1h/
wash/23h wait
[3H]-Inositiol phosphates
(corrected dpm/well)
35000
30000
25000
20000
15000
10000
5000
0
Basal
-8
-7
-6
-5
-4
-3
-2
Log10[Carbachol] (M)
(B)
Atropine 1h/wash/23h wait
Xanomeline & atropine 1h/
wash/23h wait
[3H]-Inositiol phosphates
(corrected dpm/well)
30000
25000
20000
15000
10000
5000
0
Basal
Log10[Carbachol] (M)
(C)
Atropine 23h
Xanomeline 1h/
wash/23h atropine
[3H]-Inositiol phosphates
(corrected dpm/well)
Basal
Log10[Carbachol] (M)

Figure 3. Effects of atropine on long-term modulation of inositol phosphates production by xanomeline in CHO cells expressing hM3 receptors. A) Cells were either untreated (closed square) or treated with 10 μM xanomeline for 1 hour followed by washing and waiting for 23 hours in control medium (open triangles). B) Cells were treated with atropine (10 μM) for 1 hour (closed circles) or treated simultaneously with atropine and xanomeline for 1 hour (open circles) followed by washing and waiting 23 hours in control medium. C) Cells were untreated (closed diamonds) or treated with xanomeline for 1 hour (open diamonds) followed by washing and incubation with atropine for 23 hours prior to washing. In all cases, the production of inositol phosphates was determined after the various pretreatments in the presence of increasing concentrations of carbachol (10 nM – 1 mM) for 1 hour at 37°C in the presence of 10 mM LiCl. Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Figure 4

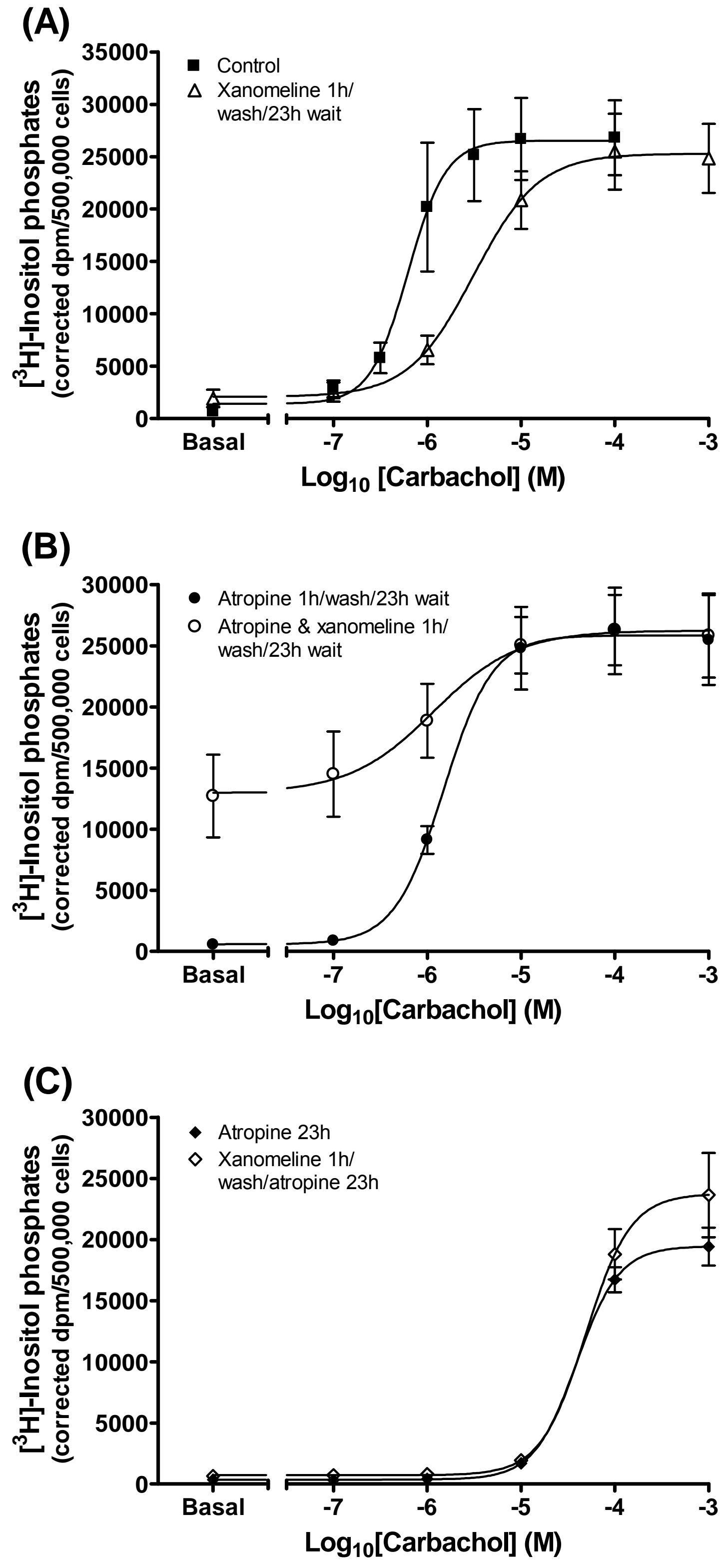


Figure 4. Effects of atropine on long-term modulation of inositol phosphates production by xanomeline in CHO cells expressing hM1 receptors. A) Cells were either untreated (closed square) or treated with 300 nM xanomeline for 1 hour followed by washing and waiting for 23 hours in control medium (open triangles). B) Cells were treated with atropine (10 μM) for 1 hour (closed circles) or treated simultaneously with atropine and xanomeline for 1 hour (open circles) followed by washing and waiting 23 hours in control medium. C) Cells were untreated (closed diamonds) or treated with xanomeline for 1 hour (open diamonds) followed by washing and incubation with atropine for 23 hours prior to washing. In all cases, the production of inositol phosphates was determined after the various pretreatments in the presence of increasing concentrations of carbachol (10 nM – 1 mM) for 1 hour at 37°C in the presence of 10 mM LiCl. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate. Data provided by Kayla De Lorme and Marianne Grant.

Figure 5

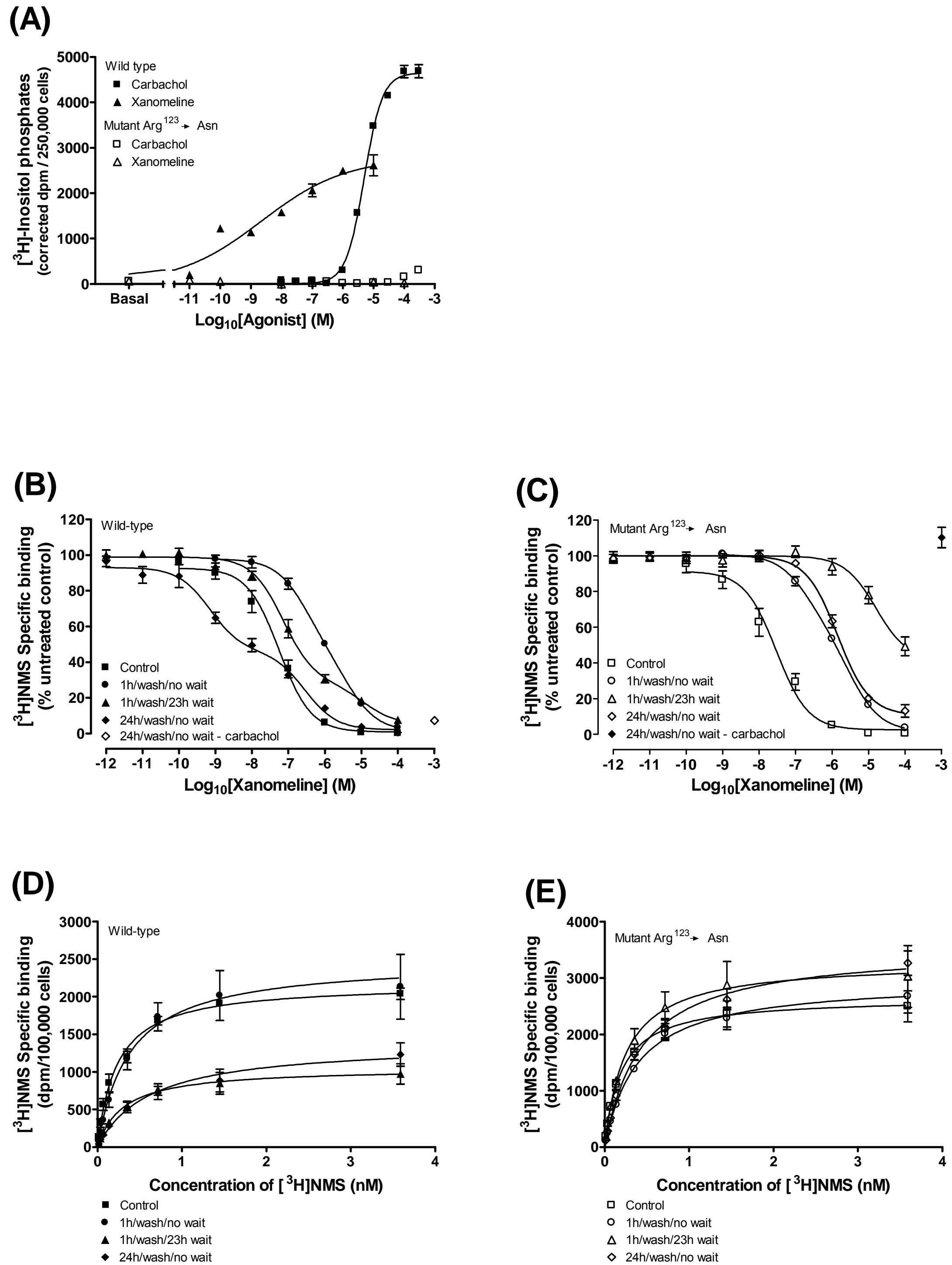


Figure 5. Effects of using a non-functional receptor mutant on function, inhibition of [^{3}H]NMS and saturation binding in CHO cells expressing rM1 receptors. A) Cells expressing wild-type (closed symbols) and mutant rM1 (open symbols) receptors were stimulated with increasing concentrations of carbachol (10 nM – 100 µM) (squares) or xanomeline (10 pM – 10 µM) (triangles) for 1 hour at 37ºC in the presence of 10 mM lithium chloride and inositol phosphate production was determined. B, D) Cells expressing wild-type rM1 receptors were treated with xanomeline for 1 hour followed by washing and used immediately (circles) or subsequent to 23 hour incubation in control media (triangles). Additional cells were treated with xanomeline for 24 hours prior to washing (diamonds). B) Untreated cells were incubated concurrently with increasing concentrations of xanomeline and 0.2 nM [^{3}H]NMS or xanomeline pretreated cells were incubated with 0.2 nM [^{3}H]NMS for 1 hour at 37 ºC. D) Untreated or xanomeline pretreated cells were incubated with increasing concentration of [^{3}H]NMS (0.02 -3.5 nM) for 1 hour at 37ºC. C, E) Cells expressing the $rM1^{123}$ mutant receptor were treated the same as described for (B) and (D). For B and C individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 13100 and 11500 dpm/well for wild-type and mutant rM1 receptors, respectively. Values represent the means ± S.E. of 2-7 experiments conducted in triplicate. Data in (A) provided by Marianne Grant.

Figure 6

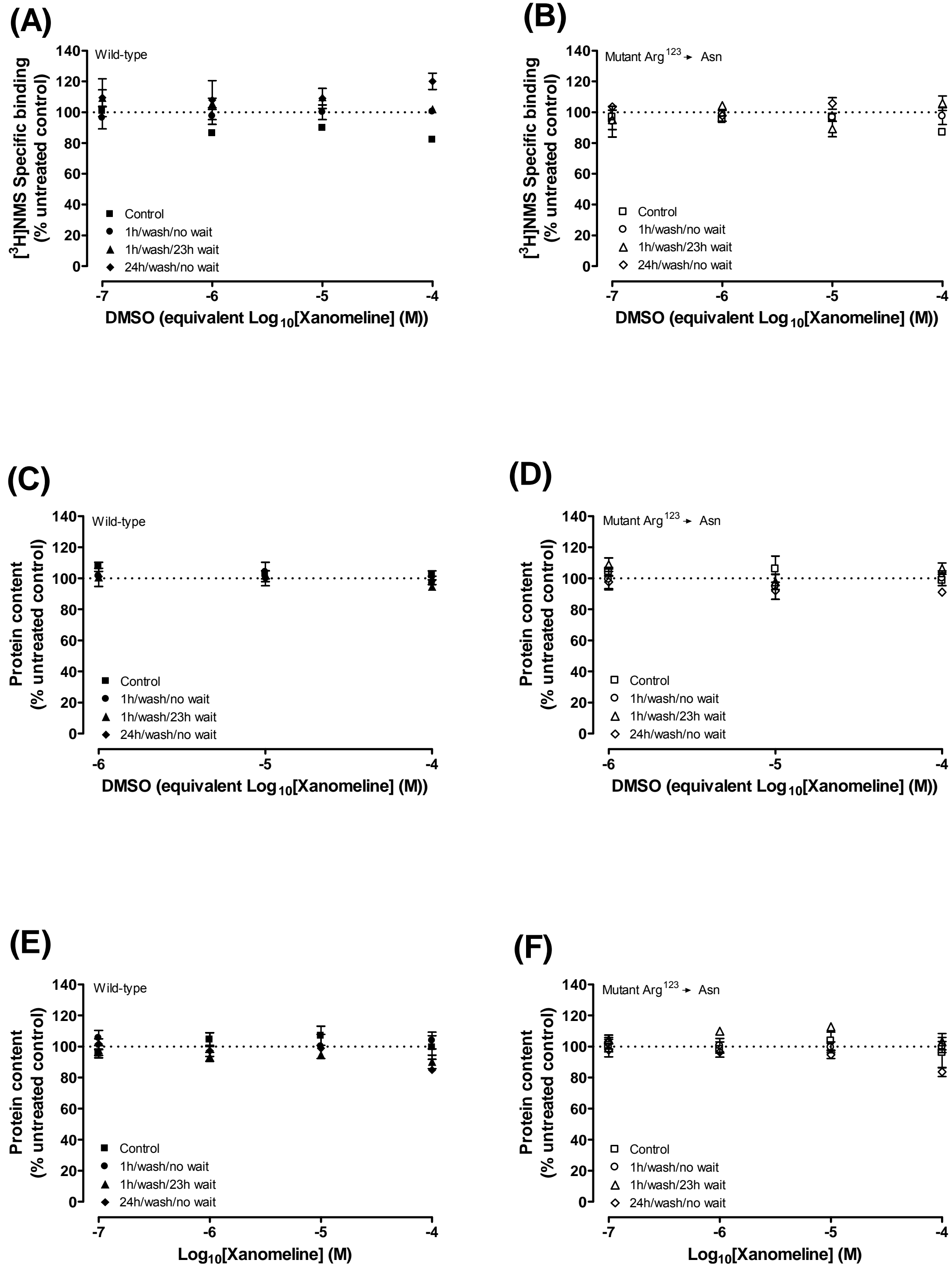


Figure 6. Control experiments to determine [^{3}H]NMS binding and protein content following xanomeline or DMSO pretreatments in CHO cells expressing rM1 receptors. Cells expressing wild-type (closed symbols) or rM1^{123} mutant receptors (open symbols) were treated with increasing concentrations of xanomeline or the corresponding concentrations of DMSO for 1 hour followed by washing and used immediately (circles) or subsequent to 23 hour incubation (triangles). Additional cells were treated with xanomeline or the corresponding concentration of DMSO for 24 hours prior to washing (diamonds). Control cells were untreated (squares). A, B) Following pretreatment with DMSO cells were incubated with 0.2 nM [^{3}H]NMS for 1 hour at 37ºC. C, D) Following pretreatment with DMSO protein content was determined using the method of Bradford (1976). E, F) Following pretreatment with xanomeline protein content was determined using the method of Bradford (Bradford, 1976). For A and B individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 9300 and 7500 dpm/well for wild-type and mutant rM1 receptors, respectively. For C and D individual experiments were normalized to control protein in the absence of DMSO treatment which had a mean of 227 and 175 µg/well for wild-type and mutant rM1 receptors, respectively. For E and F individual experiments were normalized to control protein in the absence of xanomeline treatment which had a mean of 223 and 185 µg/well for wild-type and mutant rM1 receptors, respectively. Values represent the means ± S.E. of 2-4 experiments conducted in duplicate or triplicate

Figure 7

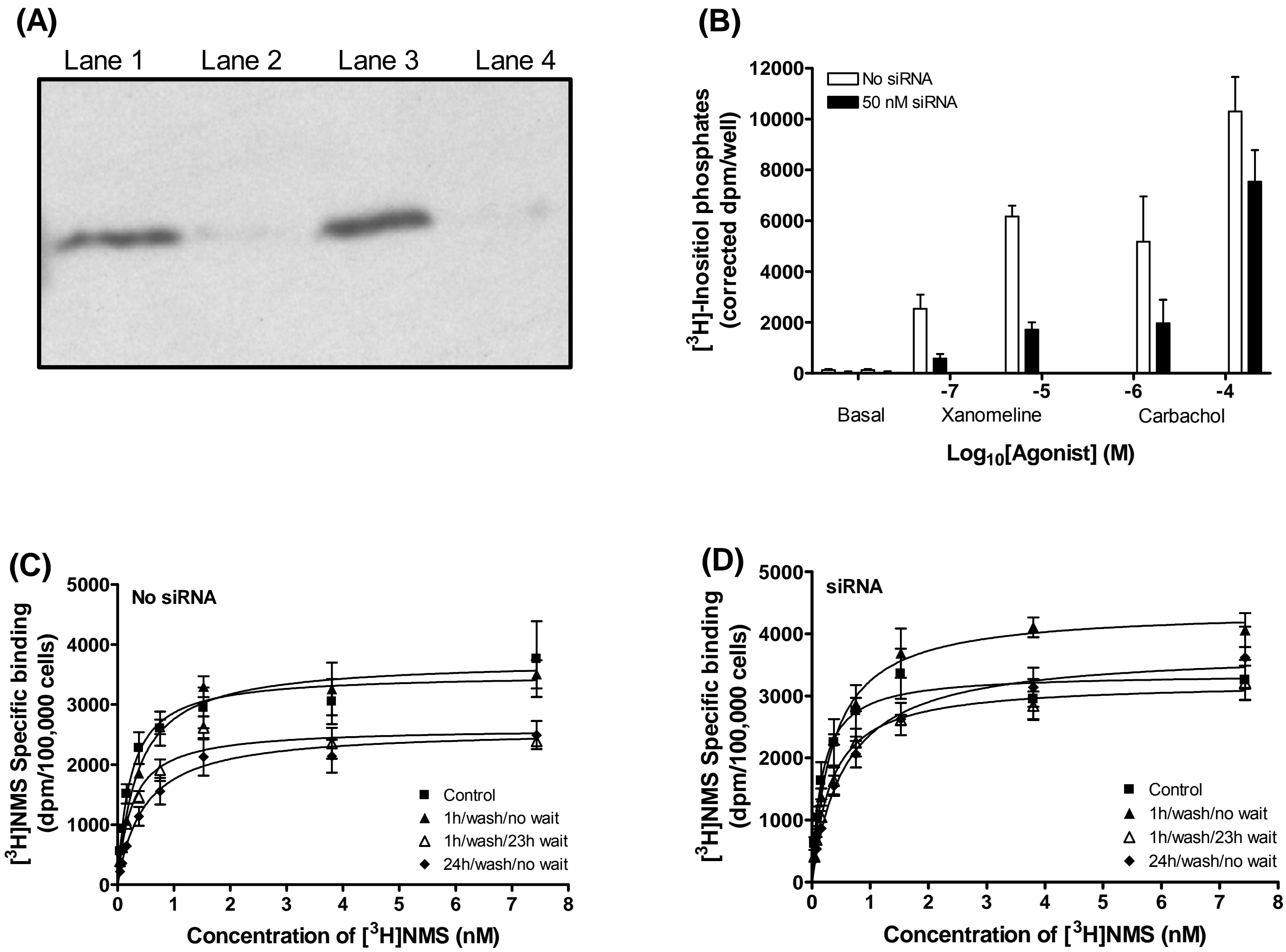


Figure 7. Effects of blocking receptor function using siRNA against G_q and G_{11} G proteins on long-term xanomeline-induced inhibition of [^{3}H]NMS binding in CHO cells expressing the hM3 receptor. A) Cells were left untreated (lane 1 & 3) or treated with 50 nM siRNA for G_q and G_{11} G proteins (lane 2 & 4). The level of $G_{q/11}$ G protein expression was determined by western blots. B) Cells were untreated (open bars) or treated with 50 nM siRNA for G_q and G_{11} G proteins (solid bars). Inositol phosphate production was determined following stimulation with two concentrations of carbachol (1 μM or 100 μM) or xanomeline (100 nM or 10 μM). C) Cells were treated with xanomeline (10 μM) for 1 hour followed by washing and used immediately (closed triangles) or after 23 hour incubation in control medium (open triangles). An additional group of cells was treated with xanomeline for 24 hours prior to washing (closed diamonds). Subsequently, untreated cells (closed squares) or pretreated cells were incubated in suspension with increasing concentrations of [^{3}H]NMS (0.01 – 7.5 nM) for 1 hour at 37°C. D) Cells were treated with 50 nM siRNA for G_q and G_{11} G proteins to inhibit receptor function then subjected to various xanomeline treatment protocols as in (C). Values represent the means ± S.E. of 3 experiments conducted in triplicate.

Figure 8

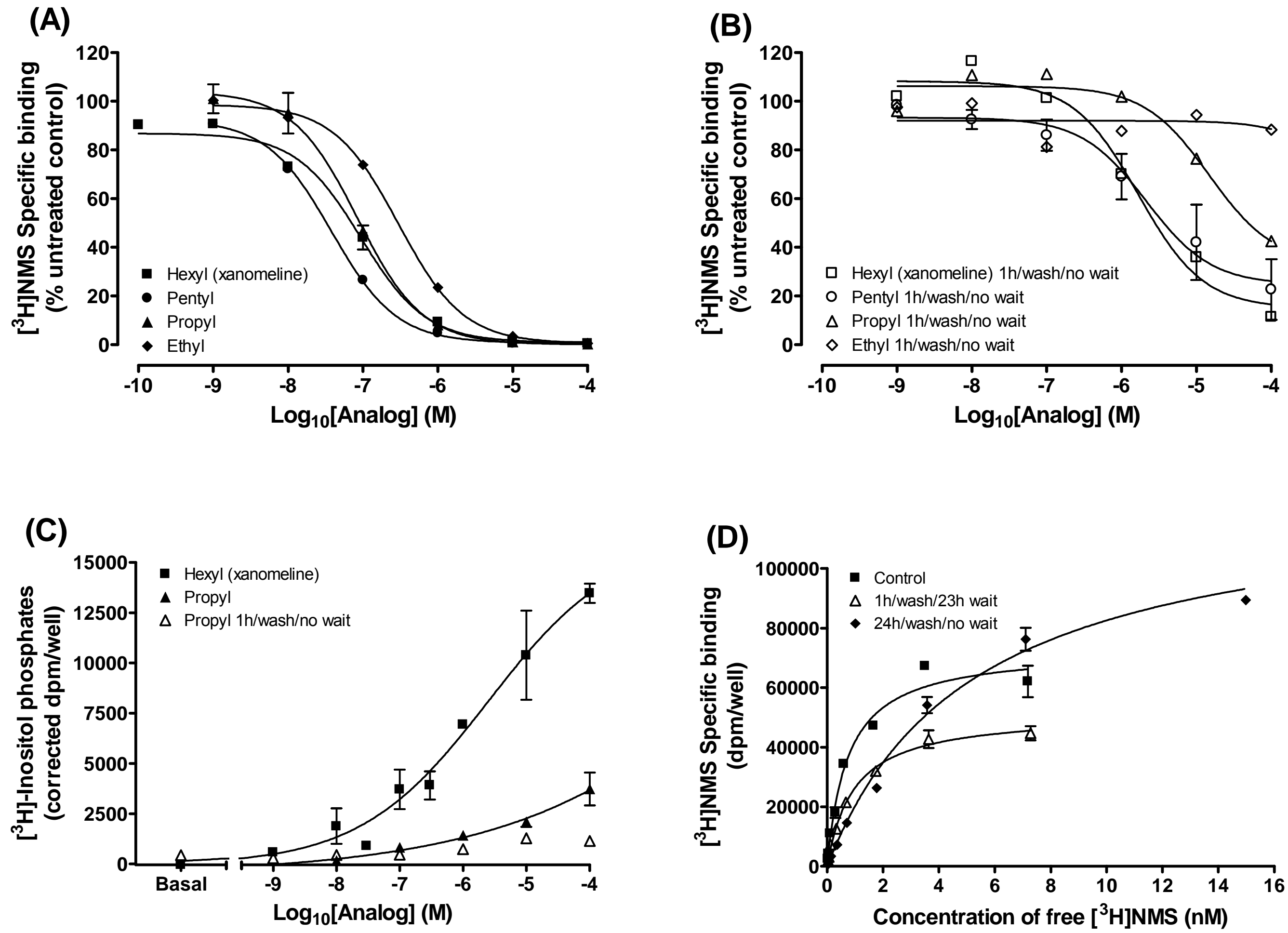


Figure 8. Effects of wash-resistant binding in the absence of receptor activation on the ability of the propyl analog of xanomeline to initiate long-term effects on saturation binding parameters in CHO cells expressing hM3 receptors.
The xanomeline analogs that were studied are hexyl (xanomeline; squares), pentyl (circles), propyl (triangles) and ethyl (diamonds). A) Cells were exposed to xanomeline analogs concurrently with 0.2 nM [^{3}H]NMS for 1 hour at 37ºC (closed symbols). B) Cells were pretreated with increasing concentrations of xanomeline analogs for 1 hour followed by washing (open symbols). Subsequently, cells were exposed to 0.2 nM [^{3}H]NMS for 1 hour at 37ºC. C) Cells were stimulated with increasing concentrations of xanomeline (closed squares) or the propyl analog (closed triangles) for 1 hour in the presence of 10 mM LiCl and inositol phosphate production was determined. Additional cells were pretreated with the propyl analog for 1 hour followed by washing (open triangles). The production of inositol phosphates was determined in the absence of further agonist stimulation in the presence of 10 mM LiCl. D) Cells were pretreated with 100 μM of the propyl xanomeline analog for 1 hour followed by washing and incubation in the absence of free drug for 23 hours (open triangles). An additional group of cells was treated for 24 hours with the propyl analog prior to washing (closed diamonds). Subsequently, untreated cells (closed square) or pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.02 - 15 nM) for 1 hour at 37°C. For A and B individual experiments were normalized to control binding in the absence of treatment with xanomeline analogs which had a mean of 14400 dpm/well. Values represent the means ± S.E. of 3 experiments conducted in duplicate.

Table 1. Effects of xanomeline pretreatment and washout in the absence or presence of atropine on [^{3}H]NMS saturation binding and functional parameters of carbachol stimulation in CHO cells expressing hM1 or hM3 receptors. Cells were pretreated for 1 hour with 3 μM (M1, binding), 300 nM (M1, function) or 10 μM (M3) xanomeline in the absence or presence of 10 μM atropine followed by washing and waiting for 23 hours in the absence or presence of atropine. In the binding assay, untreated or pretreated cells were incubated with increasing concentrations of [^{3}H]NMS for 1 hour at 37ºC. In the functional assay, untreated or pretreated cells were stimulated with increasing concentrations of carbachol for 1 hour at 37°C in the presence of 10 mM lithium chloride. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	Saturation Binding Parameters		**Functional Parameters**	
	K_d [a] (nM)	**B_{max} [b]**	**pEC_{50} [c]**	**E_{max} [d]**
M1 receptor – pretreatment groups				
Control (untreated)[e]	0.31 ± 0.02	11600 ± 1200	6.1 ± 0.09	24700 ± 2200
1 h xanomeline/washout/23 h wait	0.36 ± 0.02	2400 ± 300[$]	5.4 ± 0.12[$]	23500 ± 2100
1 h atropine/washout/23 h wait	0.52 ± 0.09	19000 ± 800	5.9 ± 0.09	26600 ± 3400
1 h xanomeline & atropine/washout/23 h wait	0.55 ± 0.02	6100 ± 1300[#]	5.9 ± 0.10	25800 ± 3600
23 h atropine/washout/no wait	2.38 ± 0.31	14200 ± 2700	4.4 ± 0.07	19500 ± 1600
1h xanomeline/washout/23 h atropine/washout/no wait	2.64 ± 0.49	13500 ± 800	4.3 ± 0.04	23800 ± 3500
M3 receptor – pretreatment groups				
Control (untreated)[e]	0.38 ± 0.05	66000 ± 11000	6.0 ± 0.18	29600 ± 2700
1 h xanomeline/washout/23 h wait	0.47 ± 0.03*	27000 ± 5500*	5.3 ± 0.07*	26100 ± 2300
1 h atropine/washout/23 h wait	0.58 ± 0.05	67000 ± 7400	5.6 ± 0.17	24200 ± 2000
1 h xanomeline & atropine/washout/23 h wait	0.66 ± 0.07	40000 ± 7400[†]	5.3 ± 0.16	24600 ± 1600
23 h atropine/washout/no wait	3.20 ± 0.39	68000 ± 11800	4.2 ± 0.01	28200 ± 3200
1h xanomeline/washout/23 h atropine/washout/no wait	3.25 ± 0.71	67000 ± 17000	4.2 ± 0.09	21100 ± 830

Table 1 cont.

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

[b] Maximal cell-surface receptor density (dpm/100,000 cells (M1) or dpm/well (M3)).

[c] Negative logarithm of midpoint (potency) parameter.

[d] Maximal response. Values are expressed as dpm/500,000 cells (M1) or dpm/well (M3) after correcting for standard recovery.

[e] Control experiments were conducted on untreated cells in the binding assay and untreated cells were stimulated with carbachol in the functional assay.

[$] Unpaired t-test detected a significant difference ($p < 0.05$) in K_d, B_{max}, pEC_{50} or E_{max} where indicated between the 1h/wash/23h wait xanomeline pretreated group compared to control at the M1 receptor.

[#] Unpaired t-test detected a significant difference ($p < 0.05$) in K_d, B_{max}, pEC_{50} or E_{max} where indicated between the 1h xanomeline and atropine/wash/23h wait pretreated group compared to 1h atropine/wash/23h wait treatment at the M1 receptor.

*Paired t-test detected a significant difference ($p < 0.05$) in K_d, B_{max}, pEC_{50} or pEC_{50} where indicated between the 1h/wash/23h wait xanomeline pretreated group compared to control at the M3 receptor.

[†] Paired t-test detected a significant difference ($p < 0.05$) in K_d, B_{max}, pEC_{50} or E_{max} where indicated between the 1h xanomeline and atropine/wash/23h wait pretreated group compared to 1h atropine/wash/23h wait treatment at the M3 receptor.

No significant difference was detected between the 1h xanomeline/wash/23h atropine/wash/no wait group compared to 23h atropine/wash/no wait treatment at either the M1 or M3 receptor.

Table 2. Effects of xanomeline pretreatment on inhibition of NMS binding, saturation binding and functional parameters in wild-type and mutant rM1 receptors expressed in CHO cells. Cells were pretreated with increasing concentrations (1 pM - 100μM) or 3 μM xanomeline for one hour followed by washing and used immediately or after 23 hour incubation in ligand free media. Additional cells were treated with increasing concentrations (1 pM – 100 μM) or 3 μM xanomeline for 24 hour prior to washing. In the functional assay, untreated cells were exposed to increasing concentrations of carbachol or xanomeline in the presence of 10 mM lithium chloride for one hour at 37ºC. In the [^{3}H]NMS inhibition binding assay, untreated cells were incubated simultaneously with xanomeline and 0.2 nM [^{3}H]NMS or pretreated cells were incubated with 0.2 nM [^{3}H]NMS for 1 hour at 37ºC. In the saturation binding assays, untreated or pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.02 – 3.6 nM) for 1 hour at 37ºC. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	rM1 wild-type								
	Functional parameters		**Inhibition of [^{3}H]NMS binding parameters**				**Saturation parameters**	
	pEC_{50} [a]	**E_{max}** [b]	**pIC_{50}** [c]	**pIC_{50} – H** [d]	**pIC_{50} – L** [e]	**I_{max}** [f]	**K_d** [g] **(nM)**	**B_{max}** [h]
Carbachol								
Control (untreated)	5.3	4700						
Xanomeline								
Control (untreated)	8.7	2800	7.3 ± 0.14			99 ± 0.6	0.27 ± 0.08	2200 ± 90
1 h/washout/no wait			6.0 ± 0.06$			95 ± 0.5	0.40 ± 0.04	2600 ± 400
1 h/washout/23 h wait				7.1 ± 0.16* (74 ± 1.1%)	4.6 ± 0.23*	99 ± 2.3	0.30 ± 0.04	1100 ± 150#
24 h/washout/no wait				9.4 ± 0.20* (47 ± 7.7%)	6.6 ± 0.12	97 ± 0.5	0.60 ± 0.12#	1300 ± 150

Table 2 cont.

	rM1^{123} Mutant							
	Functional parameters		**Inhibition of [^{3}H]NMS binding parameters**				**Saturation parameters**	
	pEC_{50}	**E_{max}**	**pIC_{50}**	**pIC_{50} – H**	**pIC_{50} – L**	**I_{max}**	**K_d (nM)**	**B_{max} ^{h}**
Carbachol								
Control (untreated)	ND	ND						
Xanomeline								
Control (untreated)	ND	ND	7.6 ± 0.14			99 ± 0.5	0.19 ± 0.02	2700 ± 300
1 h/washout/no wait			6.0 ± 0.02$^\$$			96 ± 0.7	0.42 ± 0.10	3000 ± 380
1 h/washout/23 h wait			4.9 ± 0.23*			60 ± 7.4‡	0.26 ± 0.07	3400 ± 600
24 h/washout/no wait			5.8 ± 0.08			90 ± 2.9	0.49 ± 0.11	3600 ± 320

[a] Negative logarithm of midpoint (potency) parameter.
[b] Maximal response. Values are expressed as dpm/250,000 cells after correcting for standard recovery.
[c] Negative logarithm of the IC_{50} for binding to a single potency site.
[d] Negative logarithm of the IC_{50} for the high-potency site. Percent of binding sites shown in parentheses.
[e] Negative logarithm of the IC_{50} for the low-potency site.
[f] Maximal percent inhibition of [^{3}H]NMS binding.
[g] Equilibrium dissociation constant for [^{3}H]NMS binding.
[h] Maximal cell-surface receptor density (dpm/100,000 cells).
Statistical analysis was not performed for functional parameters (pEC_{50} or E_{max}) because the data presented are from a single experiment conducted in duplicate.
[$] Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} between the xanomeline 1h/wash/no wait group compared to their respective controls at the wild-type or mutant rM1 receptor.
*ANOVA followed by Dunnett's post-test detected a significant difference ($p < 0.05$) in pIC_{50} between the 1h/wash/23h wait or 24h/wash/no wait groups compared their respective 1h/wash/no wait group at the wild-type or mutant rM1 receptor.
[#]ANOVA followed by Dunnett's post-test detected a significant difference ($p < 0.05$) in K_d or B_{max} where indicated between the xanomeline pretreated groups compared to their respective controls at the wild-type or mutant rM1 receptor.
[‡] Significantly different ($p < 0.5$) from 100 percent as determined by ANOVA followed by Dunnett's post-test comparison.

Table 3. Effects of blocking receptor function using siRNA against G_q and G_{11} G proteins on [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors assayed in suspension. Cells were untreated or treated with 50 nM siRNA for G_q and G_{11} G proteins. Subsequently, cells were treated with 10 μM xanomeline for 1 hour followed by washing and used immediately or after 23 hour incubation in control media. An additional group of cells was treated with xanomeline for 24 hours prior to washing. Cells were then incubated in suspension with increasing concentrations of [^{3}H]NMS (0.04 – 7.4 nM) at 37ºC for 1 hour. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	Control (no siRNA)		siRNA	
Xanomeline pretreatment group	**K_d [a] (nM)**	**B_{max} [b]**	**K_d (nM)**	**B_{max}**
Control (untreated)	0.22 ± 0.05	3500 ± 500	0.22 ± 0.06	3400 ± 300
1 h/washout/no wait	0.33 ± 0.04	3800 ± 400	0.40 ± 0.07	4400 ± 300
1 h/washout/23 h wait	0.24 ± 0.02	2600 ± 100	0.36 ± 0.01	3200 ± 200
24 h/washout/no wait	0.48 ± 0.12	2600 ± 300	0.63 ± 0.13*	3700 ± 500

[a] Equilibrium dissociation constant of [^{3}H]NMS binding.
[b] Cell-surface receptor density (dpm/100,000 cells).
*ANOVA followed by Dunnett's post-test detected a significant difference ($p < 0.05$) in K_d or B_{max} where indicated between the siRNA-pretreated groups compared with siRNA treated control.
No significant difference was detected between no siRNA-pretreated groups compared to no siRNA treated control.

Table 4. Effects of xanomeline analogs on inhibition of [^{3}H]NMS binding and functional parameters in CHO cells expressing hM3 receptors. Cells were treated with increasing concentration of the xanomeline analogs (1 nM – 100 μM) for 1 hour followed by washing. In the binding assay, untreated cells were incubated concurrently with 0.2 nM [^{3}H]NMS and xanomeline analogs or pretreated cells were incubated with 0.2 nM [^{3}H]NMS for 1 hour at 37ºC. In the functional assay, untreated cells were stimulated with increasing concentration of the analogs and pretreated cells were not further stimulated in the presence of 10 mM lithium chloride for 1 hour at 37°C.

	Hexyl (xanomeline)				Pentyl		Propyl				Ethyl	
	Binding parameters		Functional parameters		Binding parameters		Binding parameters		Functional parameters		Binding parameters	
	pIC_{50} [a]	I_{max} [b]	pEC_{50} [c]	E_{max} [d]	pIC_{50}	I_{max}	pIC_{50}	I_{max}	pEC_{50}	E_{max}	pIC_{50}	I_{max}
Control (untreated)	7.0 ± 0.02	99 ± 0.2	6.1 ± 0.24	14500	7.4	99	7.1 ± 0.01	100 ± 0.1	3.4	6500	6.6	99
1h /wash/no wait	5.8	85			5.9	87	5.1 ± 0.26	66 ± 0.8	ND	ND	ND	ND

[a] Negative logarithm of the IC_{50} for binding to a single potency site.
[b] Maximal percent inhibition of [^{3}H]NMS binding.
[c] Negative logarithm of midpoint (potency) parameter.
[d] Maximal response. Values are expressed as dpm/well after correcting for standard recovery.
ND – parameter values were not determined
Statistical analyses were not performed because data are the results of 1-2 experiments conducted in duplicate or triplicate.

Table 5. Effects of treatment with the propyl xanomeline analog on [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors. Cells were treated with 100 μM of the propyl analog for 1 hour followed by washing and used after 23 hour incubation in control media or treated with the propyl analog for 24 hours prior to washing. Untreated or pretreated cells were then incubated with increasing concentrations of [^{3}H]NMS (0.03 – 7.2 nM) for 1 hour at 37ºC. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

Pretreatment group	K_d [a] (nM)	B_{max} [b]
Control (untreated)	0.74 ± 0.10	73400 ± 4900
1 h/washout/23h wait	0.93 ± 0.06	51500 ± 3100
24 h/washout/no wait	5.73 ± 0.74*	133100 ± 10500*

[a] Equilibrium dissociation constant of [^{3}H]NMS binding.
[b] Cell-surface receptor density (dpm/well).
*ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in K_d and B_{max} where indicated between the propyl analog-pretreated groups compared with control.

CHAPTER 6: Mechanisms of the long-term effects of xanomeline wash-resistant binding.

Introduction

Acute wash-resistant binding of xanomeline to muscarinic receptors leads to delayed changes in receptor binding and functional properties. Previous research has shown that receptor regulation following prolonged exposure to conventional agonists can occur through various mechanisms such as internalization or down-regulation of the receptor or uncoupling of the receptor from the G protein (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990). In addition these changes have been shown to occur in a time-dependent manner. It has been shown that brief treatment of cells that express muscarinic receptors with carbachol results in a decrease in binding of hydrophilic ligands (e.g., [^{3}H]NMS), but not lipophilic ones (e.g. [^{3}H]QNB) (Maloteaux et al., 1983). These ligands are capable of binding to only cell surface receptors and both cell surface and internalized receptors, respectively. Thus, brief treatment with carbachol induces receptors to internalize without being down regulated. In contract long-term carbachol treatment causes receptor down regulation.

Many factors are involved in agonist-induced receptor regulation, including the preferential signal pathway to which the receptor is coupled, agonist efficacy and temperature. Both the M1 and M3 receptors are coupled to the $G_{q/11}$ G proteins which lead to stimulation of phospholipase C (Volpicelli & Levey, 2004). In contrast, the M2 and M4 receptors are coupled to the $G_{i/o}$ G proteins that lead to the inhibition of adenylate cyclase (Eglen, 2005). This may influence their susceptibility to regulation by agonists. Receptor regulation is also proportional to the relative efficacy of agonists at a given receptor. However, it should be noted that receptor overexpression in transfected cells could endow a partial agonist with full efficacy (Kenakin, 1997; Wood et al., 1999). It has also been shown that decreasing the temperature inhibits receptor function and internalization (El-Fakahany & Richelson, 1980; Maloteaux & Hermans, 1994).

Following prolonged exposure, xanomeline has been shown to result in a decrease in receptor expression and antagonism of the functional response to subsequent addition of agonists. The changes in the long-term responses appear to be similar at the M1 and M3 receptors despite the fact that xanomeline is a full agonist at the M1 receptor, but a partial agonist at the M3 receptor. Thus, experiments were designed to: 1) differentiate the effects of xanomeline pretreatment on receptor internalization and down regulation; 2) test the effects of changing receptor expression level on xanomeline effects; 3) further assess the role of receptor signaling in the ability of brief pretreatment with xanomeline to induce delayed receptor regulation.

Results

Comparison of the effects of brief and long-term exposure of cells to xanomeline on [^{3}H]QNB and [^{3}H]NMS binding at the M3 and M1 receptors

Prolonged agonist exposure can lead to receptor internalization, down-regulation or uncoupling of the receptor from its G protein (Dohlman et al., 1991; El-Fakahany & Cioffi, 1990). The current set of experiments was conducted to differentiate the long-term effects of xanomeline on internalization and down-regulation of the M3 and M1 receptors. These receptors both couple to $G_{q/11}$ G proteins (Volpicelli & Levey, 2004), resulting in the possibility that the mechanism of regulation may be similar between these two subtypes. QNB is a lipophilic muscarinic receptor antagonist that is able to bind to both cell-surface and internalized (but intact) receptors (Ehlert et al., 1981; Vickroy & Malphurs, 1994). In contrast, NMS (a permanently-charged quaternary amine) is only able to bind to receptors expressed on the cell surface (Feigenbaum & El-Fakahany, 1985). 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 nM [^{3}H]QNB or 2.8 nM [^{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 expressing the M3 receptor, inclusion of xanomeline in the binding assay resulted in complete concentration-dependent inhibition of binding of both radioligands with similar potency (Fig. 1; Table 1). Cells pretreated with xanomeline for one minute (Fig. 1 a, b) or one hour (Fig. 1 c, d) followed by washing and immediate use in the binding assay showed wash-resistant xanomeline binding that exhibited a significantly ($p < 0.05$) lower potency against either radioligand compared to when xanomeline was included in the binding assay (Table 1). Additionally, inhibition of [^{3}H]NMS binding was incomplete following this treatment. When cells were pretreated with xanomeline for one minute or one hour followed by washing and waiting 24 (Fig. 1 a, b) or 23 (Fig. 1 c, d) hours, respectively, 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 for one hour xanomeline pretreatment. Noteworthy, inhibition of binding under these treatment conditions was incomplete for both radioligands resulting in a maximal inhibition of approximately 35-45% (Table 1).

Additional groups of cells were incubated with increasing concentrations of xanomeline for 24 hours 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 40 percent of the binding sites for both radioligands (Fig. 1). The apparent high-potency site under these

conditions demonstrated a markedly lower IC_{50} 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 1). Once again, the inhibition of binding was incomplete for both radioligands under these conditions (Table 1).

When parallel experiments were conducted in cells expressing the M1 receptor similar results were obtained for the acute xanomeline treatments. When cells were exposed to increasing concentrations of xanomeline in the concurrent presence of [^{3}H]QNB or [^{3}H]NMS there was a concentration dependent decrease in binding that resulted in complete inhibition of either radioligand binding and similar potencies of xanomeline (Fig. 2). When cells were exposed to xanomeline for one minute (Fig. 2 a, b) or one hour (Fig. 2 c, d) followed by washing and immediate use there was a concentration-dependent decrease in radioligand binding, however, inhibition was incomplete in all cases (Table 1). It should be noted that the inhibition of [^{3}H]QNB binding following one minute pretreatment and washing with xanomeline could not be determined because the binding curve was still decreasing at the highest concentration tested. However, due to technical limitations with the vehicle xanomeline was dissolved in (DMSO) higher concentrations could not be used. Additionally, all acute treatment groups resulted in a significantly ($p < 0.05$) lower potency compared to when xanomeline was continuously present in the binding assay (Table 1). When cells were treated with xanomeline for one minute or one hour followed by washing and waiting 24 (Fig. 2 a, b) or 23 (Fig. 2 c, d) hours, respectively, there was once again a concentration-dependent decrease in radioligand binding that was complete for the one-minute pretreatments, but incomplete for the one-hour pretreatments (Table 1). In all cases there was a significant ($p < 0.05$) increase in the potency compared to the respective acute wash-resistant effects.

In contrast to the results obtained at the M3 receptor, when cells expressing the M1 receptor were exposed to xanomeline for 24 hours prior to washing there was a monophasic decrease in radioligand binding, with similar potencies for both radioligands (Fig. 2). In both cases, the potencies were significantly ($p < 0.05$) higher compared to cells that were briefly treated with xanomeline. Again, the inhibition of radioligand binding was incomplete for both radioligands (Table 1).

Effects of wash-resistant binding in the absence of receptor activation on the ability of xanomeline to mediate long-term effects on saturation binding at the M3 receptor

Previous literature has shown that wash-resistant inhibition of [^{3}H]NMS binding by xanomeline occurs with markedly lower potency at 4°C than at 37°C at the M1 receptor (Jakubik et al., 2004). Furthermore, functional assays using cells expressing the M3 receptor demonstrated lack of activation of the receptor by 10 or 100 μM xanomeline at 4°C when it was present for either one or two hours (Fig. 3 a). Therefore, experiments were conducted at 4°C to further demonstrate the need for receptor activation to obtain the long-term effects of xanomeline at the M3 receptor. Increasing concentrations of [^{3}H]NMS were used to determine cell-surface receptor density and radioligand affinity. Exposure of cells to xanomeline for one hour 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 binding or radioligand affinity compared to the 4°C control (Fig. 3 b). However, when cells were exposed to 10 or 100 μM xanomeline at 4°C for 24 hours prior to washing there was a significant ($p < 0.05$) concentration-dependent decrease in [^{3}H]NMS radioligand affinity in the absence of a change in cell-surface receptor density compared to the 4°C control (Fig. 3 b). Results are summarized in Table 2.

Time course of the switch of xanomeline pharmacological profile from an agonist to an antagonist at the M3 and M1 receptors

We have shown that the presence of acute wash-resistant xanomeline binding results in an increase in basal levels of inositol phosphate production at both the M3 (Chapter 4 Fig. 3) and M1 (Chapter 4 Fig. 4) receptor. However, inositol phosphate levels were no longer elevated following prolonged waiting in control media (Chapter 4 Fig. 8 & Fig. 9). Similar results were obtained in both cases when cells were incubated with xanomeline for 24 hours prior to washing. To determine the time course of the reversal of receptor stimulation by wash-resistant xanomeline binding, cells expressing the M3 receptor were treated with xanomeline for one hour followed by washing and waiting for different durations. The initial receptor activation following pretreatment for one hour and washing subsided rapidly upon further incubation in control media, approaching control levels after 3 hours (Fig. 4 c). As can be seen in Fig. 4 d, a similar response profile was observed when xanomeline was present for increasing durations of time prior to washing (30 minutes to 24 hours), although the time-dependent reversal of persistent receptor activation occurred at a slower rate.

Xanomeline has been shown to antagonize the functional response to carbachol following washing and prolonged incubation in control media at the M3 receptor (Chapter 4 Fig. 8). Further experiments were conducted to determine the time dependence of development of xanomeline antagonistic activity at the M3 receptor. A reduction in the ability of carbachol to elicit a response was observed immediately following xanomeline treatment for one hour. This effect was maintained at a steady state upon further incubation of pretreated and washed cells in control media for various time points up to 23 hours (Fig. 4 c). In contrast, a time-dependent reduction in the ability of carbachol to

stimulate a response was observed when cells expressing the M3 receptor were treated with xanomeline for 30 minutes to 24 hours followed by washing (Fig. 4 d).

Parallel experiments were conducted at the M1 receptor with slightly different results. To determine the time course of the reversal of receptor stimulation by wash-resistant xanomeline, cells expressing the M1 receptor were treated with xanomeline for one hour followed by washing and waiting increasing durations of time. The initial receptor stimulation by wash-resistant xanomeline declined rapidly, reaching control levels after approximately 5 hours of incubation in ligand free media (Fig.4 a). This decrease took slightly longer than it did at the M3 receptor. In contrast, when cells were incubated with xanomeline for increasing amounts of time (30 minute to 24 hours) prior to washing, the initial receptor activation was slightly lower than when xanomeline was continually present in the functional assay. In this case, receptor activation by wash-resistant xanomeline decreased slowly over time, approaching basal levels after 16 hour incubation in control media (Fig. 4 b). This is in stark contrast to the rapid decrease in wash-resistant activation that was observed at the M3 receptor.

Similar to the M3 receptor, xanomeline has also been shown to antagonize the functional response to carbachol following washing and prolonged incubation in control media at the M1 receptor (Chapter 4 Fig. 9). Thus, further experiments were conducted to determine the time dependence of carbachol antagonism by xanomeline. The response to carbachol following xanomeline pretreatment for one hour decreased over time, reaching a maximum of approximately 50 percent inhibition after 9 hours of incubation in control media (Fig. 4 a). However, the decrease in the ability of carbachol to stimulate a functional response following xanomeline pretreatment for 30 minutes to 24 hours decreased in a time-dependent manner in parallel with the decrease in receptor stimulation by wash-resistant xanomeline (Fig. 4 b).

Effect of decreasing cell-surface receptor expression using a receptor alkylating agent at the M4 receptor

The level of receptor expression has been shown to influence binding and functional parameters. The level of receptor expression can change the response to an agonist (Kenakin, 1997). To determine if the lack of long-term regulation of xanomeline at the M2 receptor was due to a lower level of cell-surface receptor density, the level of receptor density was decreased at the M4 receptor to the same levels observed at the M2 receptor. To accomplish this, cells were treated with increasing concentrations of propylbenzyl choline mustard (PrBCM) (0.1 – 10 nM) for 15 minutes followed by washing and the binding of 6.5 nM [^{3}H]NMS was determined. There was a concentration dependent decrease in cell-surface receptor density as measured by the decrease in [^{3}H]NMS binding , with an approximately 50 percent decrease in binding occurring at 2 nM PrBCM treatment (Fig. 5 a). Thus, 2 nM PrBCM was used in further experiments. When cells expressing M4 receptors were exposed to 2 nM PrBCM for 15 minutes followed by washing and incubation in control media for 24 hours there was no longer a decrease in cell-surface receptor density compared to a no PrBCM treatment control (Fig. 5 b; Table 3). In contrast, when cells were treated with PrBCM for 15 minutes followed by washing and xanomeline treatment for one hour followed by washing and waiting 23 hours in ligand free media there was a decrease in cell-surface receptor density with an increase in radioligand affinity (Fig. 5 b). Similarly, when cells were treated with xanomeline for 24 hours subsequent to treatment with PrBCM there was a decrease in cell-surface receptor density. However, under these pretreatment conditions there was a large decrease in radioligand affinity (Fig. 5 b; Table 3).

Effect of blocking the $G_{i/o}$ G proteins on the long-term effects of xanomeline binding at the M4 receptor

To determine if $G_{i/o}$ G protein stimulation is necessary for the long-term effects of xanomeline at the M4 receptor cells were pretreated with pertussis toxin (100 ng/ml), which blocks $G_{i/o}$ G proteins, for 24 hours prior to xanomeline treatments. When cells were treated with xanomeline for one hour followed by washing and further incubation in the absence of free ligand for 23 hours there was decrease in cell-surface receptor density and no change in radioligand affinity compared to control. Similarly, when cells were treated with xanomeline for 24 hours prior to washing there was a decrease in cell-surface receptor expression, however, in this case there was a large decrease in radioligand affinity compared to control (Fig. 6). Overall, inactivation of $G_{i/o}$ G protein by pertussis toxin did not cause marked changes in receptor regulation by xanomeline (Fig. 6; Chapter 3 Fig. 19 d). Results are summarized in Table 3.

Discussion

Previous research has documented that prolonged exposure to an agonist can lead to receptor internalization, down-regulation or uncoupling from G proteins (Maloteaux & Hermans, 1994). Experiments were therefore conducted to determine whether the observed long-term effects of xanomeline are due to receptor internalization or down-regulation at the M3 and M1 receptors. These possibilities were differentiated by comparing the effects of xanomeline treatment on the specific binding of [^{3}H]QNB and [^{3}H]NMS (Fig. 1 & Fig. 2). QNB is a lipophilic antagonist that is able to bind to both cell-surface and internalized (but intact) receptors (Ehlert et al., 1981; Vickroy & Malphurs, 1994), whereas NMS (a permanently-charged quaternary amine) is only able to bind to receptors located on the cell surface (Feigenbaum & El-Fakahany, 1985). The lack of

significant differences in the effects of brief xanomeline pretreatment and washing on the binding of both ligands suggests absence of receptor internalization at either receptor subtype. Furthermore, similarities of the effects of 24 hour treatment with xanomeline on the binding profiles of [^{3}H]QNB and [^{3}H]NMS supports the notion that the effects observed in both displacement and saturation binding experiments are mainly due to receptor down-regulation rather than internalization. In addition, incomplete inhibition of binding of either radioligand across the various xanomeline pretreatments followed by washing at both receptor subtypes suggests that a portion of the cell surface receptors are not susceptible to regulation by xanomeline. Alternatively, this might be an indicator of allosteric modification of receptor conformation by xanomeline, since incomplete inhibition of radioligand binding is a hallmark of allosteric receptor modulation (Lee & El-Fakahany, 1991).

Interestingly, treatment with xanomeline for one hour followed by washing and waiting 23 hours indicated an apparent higher potency of xanomeline in decreasing [^{3}H]NMS than [^{3}H]QNB binding at the M3 receptor. An opposite profile would have been expected if a major proportion of the receptors were being internalized rather than down-regulated. 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 (Ehlert, 1988). Therefore, the effects of a given allosteric modulator are ligand specific. In contrast to the changes observed after one hour treatment followed by waiting at the M3 receptor, when cells expressing the M1 receptor were exposed to xanomeline for one minute or one hour followed by washing and waiting there was no

difference in potency of xanomeline in decreasing [^{3}H]QNB or [^{3}H]NMS binding. This suggests that the observed effects have some degree of receptor specificity.

Additional [^{3}H]NMS saturation binding experiments using cells expressing the M3 receptor were conducted at 4ºC, which has been shown to prevent receptor activation by agonists (El-Fakahany & Richelson, 1980; Maloteaux & Hermans, 1994). Treatment of cells with xanomeline for one hour at 37ºC followed by washing and 23-hour incubation at 4ºC did not result in changes in binding parameters compared to untreated control cells incubated at 4ºC. However, when cells were treated with 10 or 100 μM xanomeline for 24 hours at 4ºC prior to washing there was a decrease in radioligand affinity (Fig. 3). 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 cell-surface receptor density. Interestingly, while the effects of xanomeline on maximal cell-surface receptor density seem to be dependent on receptor activation at the orthosteric site, xanomeline's effects on radioligand affinity are not. This differential effect on cell-surface receptor density and radioligand affinity 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.

Increases in activation of phosphoinositide hydrolysis were seen in cells briefly treated with xanomeline and then washed at both the M3 and M1 receptors. However, when pretreated and washed cells were incubated for a long period in the absence of free xanomeline, this wash-resistant receptor activation was reversed to control unstimulated levels. The same phenomenon was observed in cells treated with xanomeline for prolonged durations prior to washing. It is possible that receptor uncoupling from the G protein, internalization or down-regulation could account for these

changes (Cioffi & El-Fakahany, 1989; van de Westerlo et al., 1995). We further explored the time dependence of the development of this biphasic pharmacological profile (Fig. 4). Elevated levels of inositol phosphates that occur during one hour xanomeline treatment followed by washing rapidly decline within the first three hours of incubation in control media in cells expressing the M3 receptor. The profile at the M1 receptor was similar, except the return to basal levels occurred after 5 hour incubation. A similar decline in wash-resistant activation was observed when M3 expressing cells were treated with xanomeline for 30 minutes to 24 hours followed by washing, just prior to performing the receptor functional assay. In contrast, the decline in wash-resistant activation following prolonged treatment with xanomeline occurred more slowly for cells expressing the M1 receptor. The discrepancy between the M3 and M1 receptors may be because xanomeline is a full agonist at the M1 receptor and thus needs fewer functional receptors to still be able to stimulate a response.

Reversal of xanomeline wash-resistant activation of the receptor is accompanied by attenuation of receptor activation by carbachol (Fig. 4). Noteworthy, while there was complete reversal of wash-resistant receptor activation by xanomeline following one hour pretreatment and washing at both receptor subtypes, its antagonistic effects on carbachol reached a maximum of only approximately 25 percent at the M3 receptor. However, the antagonist effect at the M1 receptor was greater reaching approximately a 50 percent decrease in the ability of carbachol to stimulate a response. In contrast, the ability of carbachol to elicit a response following exposure of cells expressing the M3 receptor to xanomeline for 30 minutes to 24 hours followed by washing decreases as the time of xanomeline exposure increases, with a maximal decrease in inositol phosphate production of approximately 67 percent. The response was similar at the M1 receptor; however, the maximal decrease in stimulation was approximately 85 percent. The

higher magnitude of the antagonistic effects of the latter treatment protocol on the response to carbachol might be due to its demonstrated combined effect on receptor number and affinity.

In our experiments, we detected more marked effects of xanomeline on M4 versus M2 receptors. However, the higher level of expression of M4 receptors complicates interpretation of these data. Receptor overexpression has been shown to result in partial agonists appearing to be full agonists in the expression system (Christopoulos & El-Fakahany, 1999; Kenakin, 1997; Wood et al., 1999). Thus, experiments were undertaken to determine if decreasing receptor density using an alkylating agent (PrBCM) at the M4 receptor changes the ability of xanomeline to induce long-term changes. Although receptor availability was decreased by approximately 50 percent prior to xanomeline pretreatments, receptor density in untreated cells had recovered to that of non-alkylated cells 24 hour later. Interestingly, xanomeline pretreatment following treatment with PrBCM still resulted in a decrease in cell-surface receptor density following either long-term xanomeline pretreatment, similar to the decrease observed with xanomeline treatment alone (Chapter 3 Fig. 19). This suggests that an initial decrease in receptor density is not significant in influencing the long-term effects of xanomeline. However, since cell-surface receptor density returned to untreated levels it is possible that the decrease in receptor density did not occur for long enough to detect changes in binding properties due to decreased receptor density.

We have previously demonstrated that long-term receptor regulation at the M1 and M3 receptor subtypes appears to be dependent on receptor function. Blocking function by a mutation in the $G_{q/11}$ G protein binding domain at the M1 receptor (Chapter 5 Fig. 5) or blocking function using siRNA for $G_{q/11}$ G proteins at the M3 receptor (Chapter 5 Fig. 7) results in the long-term effects of xanomeline on cell-surface receptor

density no longer being apparent. Interestingly, when cells expressing the M4 receptor were treated with pertussis toxin, which blocks the $G_{i/o}$ G protein (Eglen et al., 1987), there was no change in the binding profile following xanomeline pretreatments compared to xanomeline treatment in the absence of G protein blockade (Fig. 6). This suggests that the long-term regulation of xanomeline at the M4 receptor is mediated by a means other than $G_{i/o}$ G proteins.

From the combined results of the two previously discussed experiments we propose that the lack of long-term effects of xanomeline at the M2 receptor is due to marked attenuation of receptor response to xanomeline. It is also important to consider that since xanomeline is able to bind, but stimulates a minimal if any functional response, it may have antagonistic properties. Furthermore, this suggests that receptor regulation in response to xanomeline occurs through different mechanisms depending on the receptor subtype.

In summary, prolonged exposure to xanomeline has been shown to lead to changes in receptor regulation. These changes appear to be the result of receptor down-regulation rather than internalization. Long-term receptor regulation is also dependent on a functional response to xanomeline. At both the M1 and M3 receptors this modulation occurs in a time-dependent manner. Furthermore, we have provided evidence to suggest that the long-term modulation of the receptor by xanomeline occurs through different mechanisms at the M1 and M3 receptors compared to the M2 and M4 receptors.

Figure 1

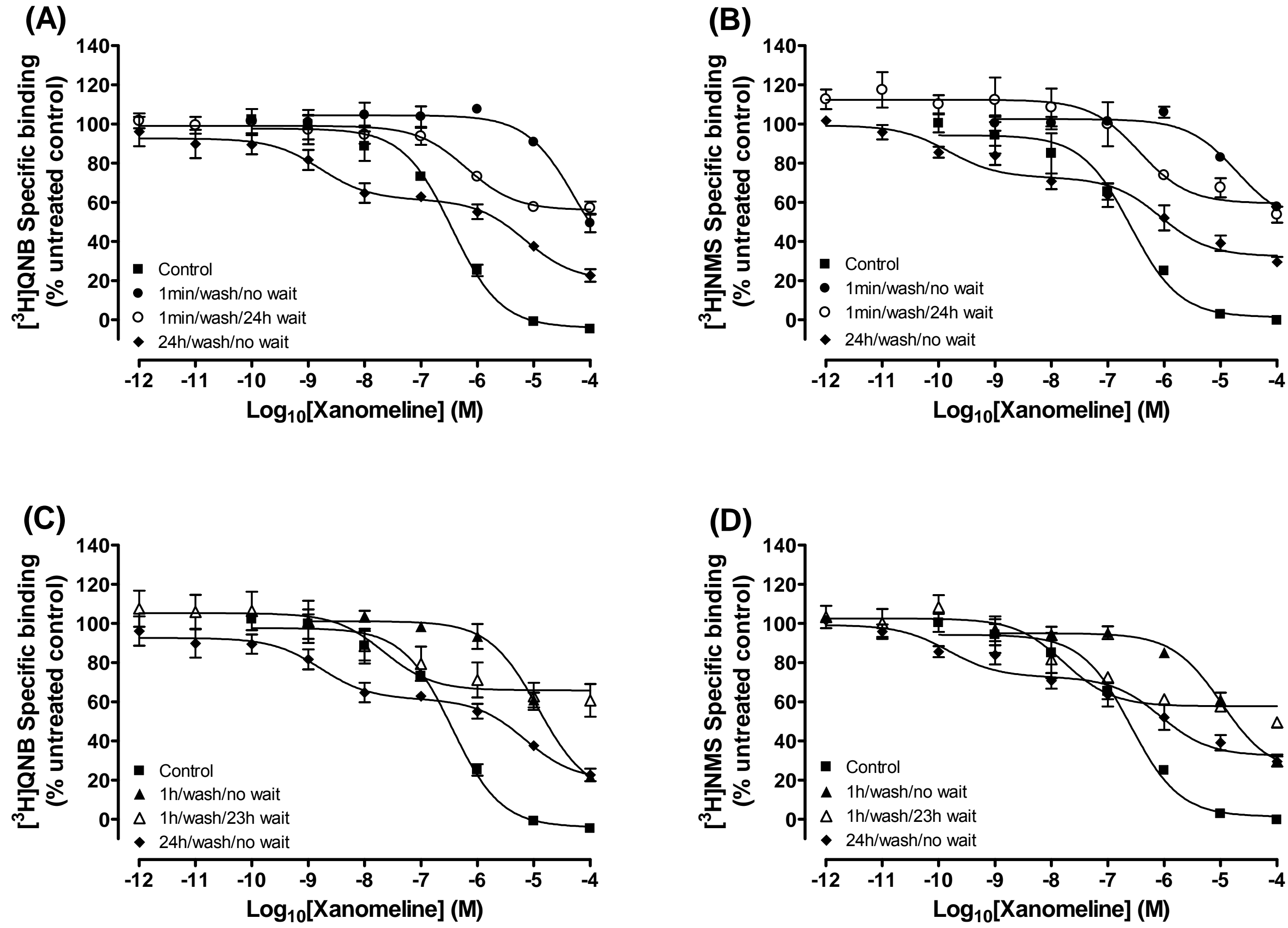


Figure 1. Comparison of the effects of xanomeline treatment on the specific binding of [^{3}H]QNB and [^{3}H]NMS in CHO cells expressing hM3 receptors. A, B) Cells expressing the M3 receptor were pretreated with increasing concentrations of xanomeline for 1 minute followed by washing and immediate use (closed circles) or subsequent to 24 hour incubation in ligand free media (open circles). Additional cells were treated with increasing concentrations of xanomeline for 24 hours prior to washing (closed diamonds). C, D) Cells were pretreated with increasing concentrations of xanomeline for 1 hour, washed and used immediately (closed triangles) or after incubation in control medium for 23 hours (open triangles). An additional group of cells was pretreated with xanomeline for 24 hours prior to washing (closed diamonds). A, C) The binding of 1.4 nM [^{3}H]QNB was determined in untreated cells in the concurrent presence of xanomeline (closed squares) or following various xanomeline pretreatments for 1 hour at 37ºC. B, D) Cells were treated the same as (A, C) except 2.9 nM [^{3}H]NMS was used in place of QNB. Non-specific binding was determined in the presence of 10 μM atropine. Individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 39500 and 56800 dpm/well for QNB and NMS, respectively. Values represent the means ± S.E. of 3-4 experiments conducted in triplicate.

Figure 2

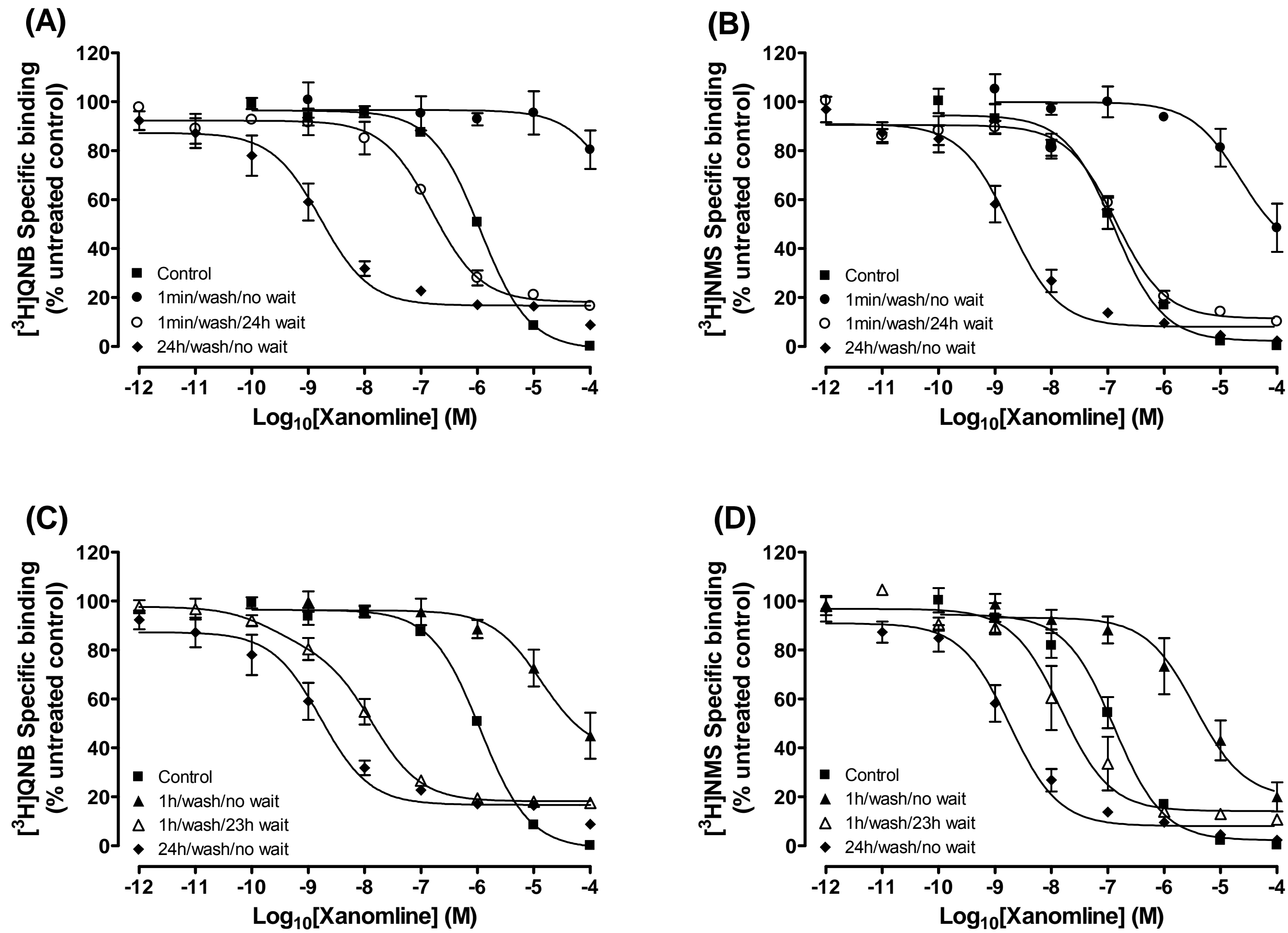


Figure 2. Comparison of the effects of xanomeline treatment on the specific binding of [^{3}H]QNB and [^{3}H]NMS in CHO cells expressing hM1 receptors. A, B) Cells expressing the M1 receptor were pretreated with increasing concentrations of xanomeline for 1 minute followed by washing and immediate use (closed circles) or subsequent to 24 hour incubation in ligand free media (open circles). Additional cells were treated with increasing concentrations of xanomeline for 24 hours prior to washing (closed diamonds). C, D) Cells were pretreated with increasing concentrations of xanomeline for 1 hour, washed and used immediately (closed triangles) or after incubation in control medium for 23 hours (open triangles). An additional group of cells was pretreated with xanomeline for 24 hours prior to washing (closed diamonds). A, C) The binding of 1.4 nM [^{3}H]QNB was determined in untreated cells in the concurrent presence of xanomeline (closed squares) or following various xanomeline pretreatments for 1 hour at 37ºC. B, D) Cells were treated the same as (A, C) except 2.9 nM [^{3}H]NMS was used in place of QNB. Non-specific binding was determined in the presence of 10 μM atropine. Individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 57500 and 80900 dpm/well for QNB and NMS, respectively. Values represent the means ± S.E. of 2-4 experiments conducted in triplicate.

Figure 3

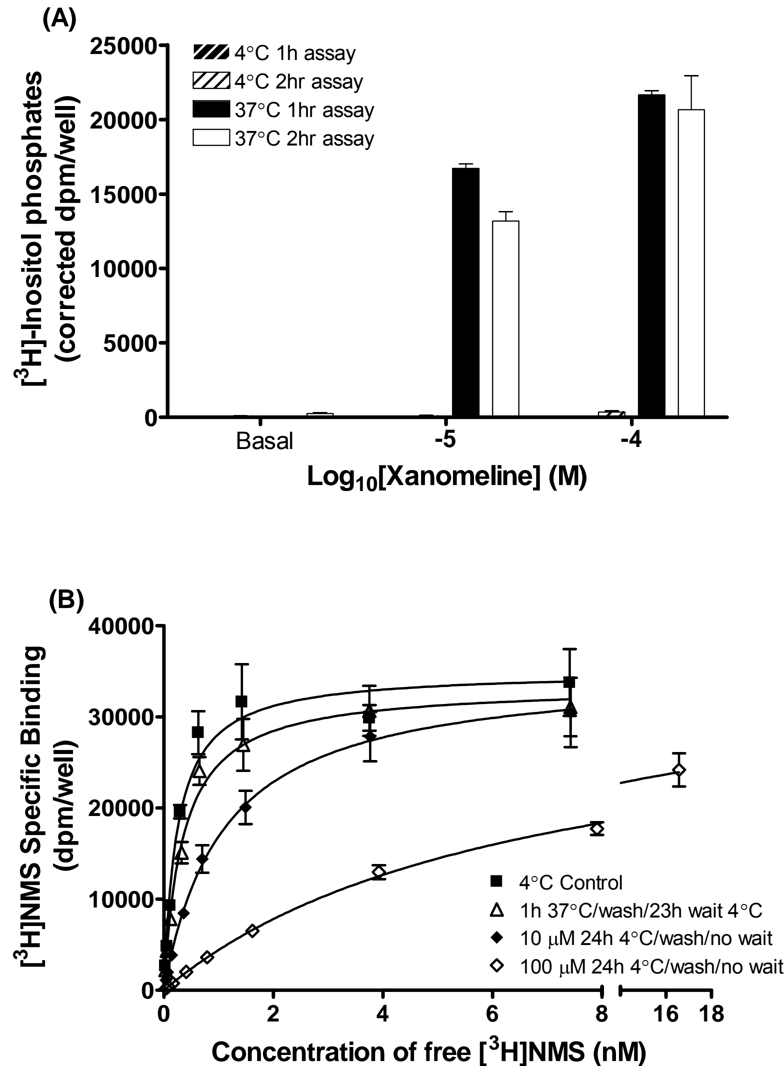


Figure 3. Effects of blocking receptor activation on the ability of xanomeline to initiate long-term effects on [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors. A) Cells were treated with 10 or 100 μM xanomeline for 1 (black bar) or 2 (white bar) hours in the presence of 10 mM lithium chloride at 4ºC (dashed) or 37ºC (solid) and the production of inositol phosphates was determined. Values represent 1 experiment conducted in triplicate. B) Cells were pretreated with 10 μM xanomeline for 1 hour at 37ºC followed by washing and incubation in the absence of free xanomeline for 23 hours at 4ºC (open triangles). Additional groups of cells were treated with 10 μM (closed diamonds) or 100 μM (open diamonds) xanomeline for 24 hours at 4ºC prior to washing. Subsequently, untreated cells incubated at 4 ºC (closed squares) or pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.02 – 16.5 nM) for 1 hour at 37°C. Non-specific binding was determined in the presence of 10 μM atropine. Values represent the means ± S.E. of 3-4 experiments conducted in duplicate.

Figure 4

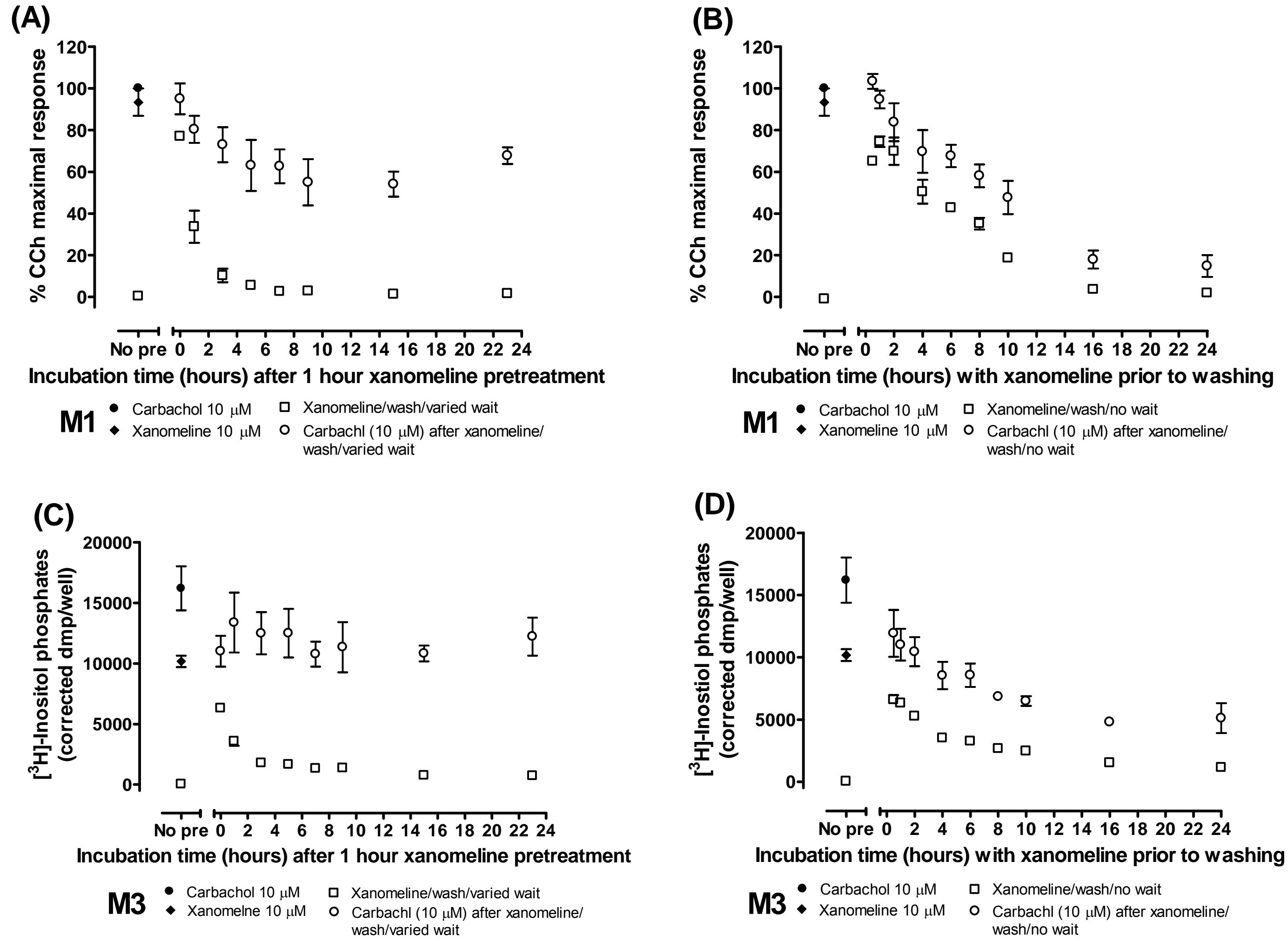


Figure 4. Time course of switching of the pharmacological profile of xanomeline from an agonist to an antagonist in CHO cells expressing hM1 or hM3 receptors. A) M1 or C) M3 cells were pretreated with 3 or 10 μM xanomeline, respectively, for 1 hour followed by washing and waiting for increasing lengths of time (0-23 hours). B) M1 or D) M3 cells were pretreated with 3 or 10 μM xanomeline, respectively, for increasing amounts of time (30 minutes -24 hours) prior to washing. Subsequently, the accumulation of inositol phosphates was determined over a 1-hour incubation period at 37ºC in the presence of lithium chloride following no further stimulation (open squares) or stimulation with 10 μM carbachol (open circles). For comparison, untreated cells were stimulated with 10 μM carbachol (closed circles) or 10 μM xanomeline (closed diamonds). No pre: untreated cells that were not exposed to xanomeline. Individual experiments at the M1 receptor were normalized to the production of inositol phosphates following 10 μM carbachol which had a mean of 31600 and 26700 dpm/well for (A) and (C), respectively. Values represent the means of 2-3 experiments conducted in triplicate.

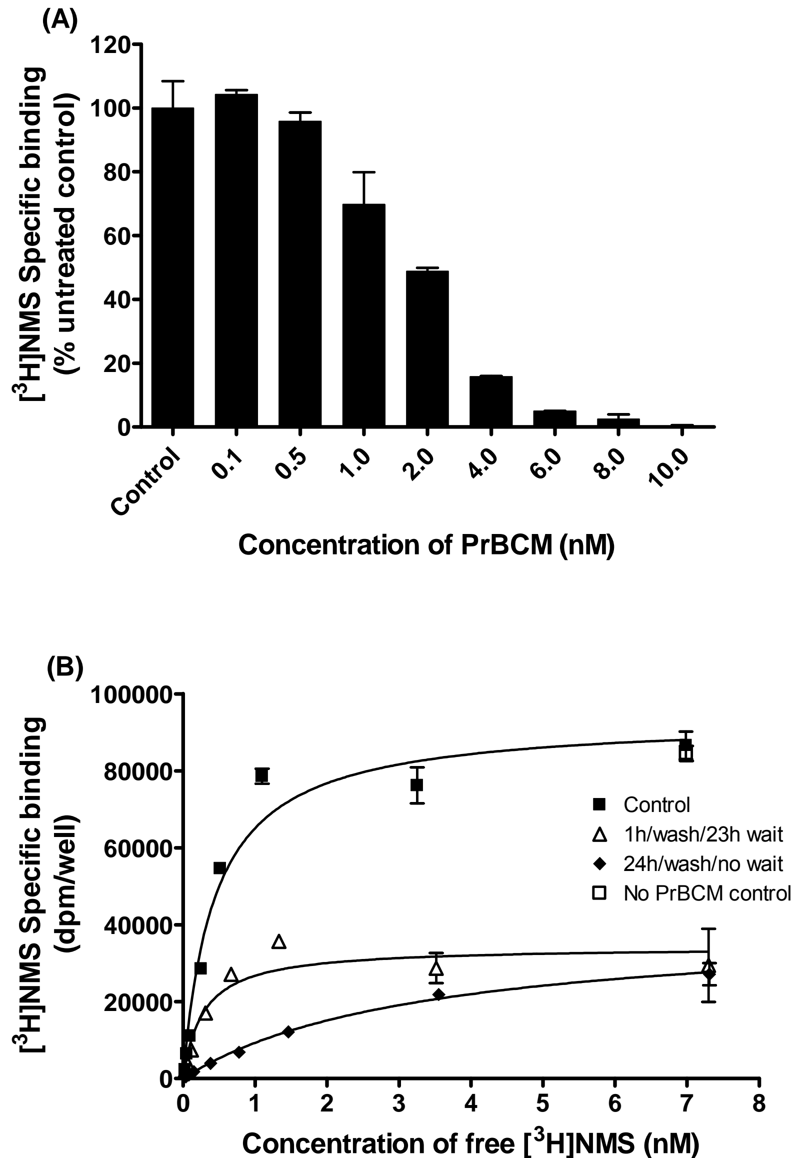


Figure 5. Effects of decreasing receptor expression using PrBCM on saturation binding parameters in CHO cells expressing hM4 receptors. A) Cells were pretreated with increasing concentration of cyclized PrBCM (0.1 – 10 nM) for 15 minutes followed by washing. Subsequently, cells were exposed to 6.5 nM [^{3}H]NMS for 1 hour at 37°C. B) Cells were pretreated with 2 nM PrBCM prior to xanomeline pretreatments. Cells were pretreated with xanomeline (10 μM) for 1 hour followed by washing and waiting 23 hours (open triangles) or treated with xanomeline for 24 hour prior to washing (closed diamonds). Cells pretreated only with PrBCM (closed squares) or xanomeline pretreated cells were exposed to increasing concentrations of [^{3}H]NMS (0.03 – 7 nM) for 1 hour at 37°C. For comparison naïve cells were exposed to the highest concentration of NMS (open square). Non-specific binding was determined in the presence of 10 μM atropine. Values represent 1 experiment conducted in triplicate.

Figure 6

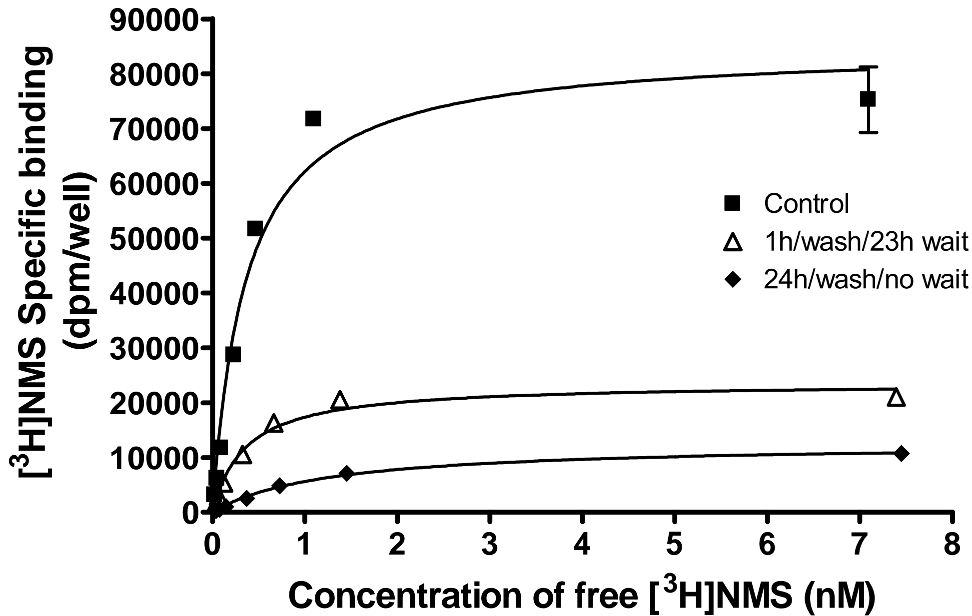


Figure 6. Effects of blocking the $G_{i/o}$ G proteins on the ability of xanomeline to induce long-term changes in CHO cells expressing the hM4 receptor. Cells were pretreated with 100 ng/ml pertussis toxin for 24 hours. Subsequently, cells were pretreated with 10 μM xanomeline for 1 hour followed by washing and 23 hour incubation in control media containing pertussis toxin (open triangles). An additional group of cells was treated concurrently with xanomeline and pertussis toxin for 24 hours prior to washing (closed diamonds). Untreated (closed squares) or xanomeline pretreated cells were then exposed to increasing concentrations of [^{3}H]NMS (0.02 – 7.5 nM) for 1 hour at 37ºC. Non-specific binding was determined in the presence of 10 μM atropine. Values represent 1 experiment conducted in triplicate.

Table 1. Inhibition of radioligand binding using a receptor-saturating concentration of [^{3}H]QNB or [^{3}H]NMS following xanomeline pretreatment and various waiting periods in CHO cells expressing hM1 or hM3 receptor subtypes. Cells were treated with increasing concentrations of xanomeline (1 pM – 100 μM) for 1 minute or 1 hour followed by washing and immediate use in the binding assay or subsequent to incubation for 24 or 23 hours, respectively, in the absence of free xanomeline. An additional group of cells was treated with xanomeline for 24 hours prior to washing. Subsequently, untreated cells were incubated concurrently with increasing concentration of xanomeline and 1.4 nM [^{3}H]QNB or 2.9 nM [^{3}H]NMS or pretreated cells were incubated with 1.4 nM [^{3}H]QNB or 2.9 nM [^{3}H]NMS for 1 hour at 37ºC. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	[^{3}H]QNB				[^{3}H]NMS			
Pretreatment group	pIC_{50} [a]	pIC_{50}-H [b]	pIC_{50}-L [c]	I_{max} [d]	pIC_{50}	pIC_{50}-H	pIC_{50}-L	I_{max}
M1 receptor								
Control (untreated)[e]	5.9 ± 0.03			102 ± 1.5	6.9 ± 0.08			98 ± 1.0
1min/washout/no wait	3.7 ± 0.57$^{\sim}$			ND	4.6 ± 0.04$^{\&}$			63 ± 10.3‡
1min/washout/24h wait	6.8 ± 0.04$^{\pm}$			82 ± 0.2	6.9 ± 0.03$^{@}$			89 ± 0.9
1 h/washout/no wait	4.8 ± 0.08$^{\#}$			62 ± 9.6‡	5.5 ± 0.20$^{\$}$			80 ± 3.6‡
1 h/washout/23 h wait	8.3 ± 0.19†			74 ± 2.0‡	7.8 ± 0.38*			84 ± 07‡
24 h/washout/no wait	9.2 ± 0.30$^{\pm†}$			72 ± 2.2‡	8.8 ±0.26$^{@*}$			88 ± 0.7‡
M3 receptor								
Control (untreated)	6.4 ± 0.11			104 ± 0.5	6.6 ± 0.16			97 ± 1.2
1min/washout/no wait	4.2 ± 0.06$^{\sim}$			82 ± 7.5‡	4.7 ± 0.16$^{\&}$			53 ± 5.9‡
1min/washout/24h wait	6.2 ± 0.19$^{\pm}$			44 ± 3.2‡	6.5 ± 0.08$^{@}$			40 ± 3.0‡
1 h/washout/no wait	4.9 ± 0.12$^{\#}$			88 ± 2.6	5.0 ± 0.09$^{\$}$			77 ± 1.9‡
1 h/washout/23 h wait	6.7 ± 0.18†			34 ± 8.0‡	7.9 ± 0.30*			43 ± 1.1‡
24 h/washout/no wait		8.8 ± 0.13$^{\pm†}$ (43 ± 3.3%)	5.1 ± 0.01	80 ± 3.2‡		9.2 ± 0.47$^{@*}$ (42 ± 3.3%)	6.0 ± 0.37	70 ± 1.5‡

Table 1 cont.

[a] Negative logarithm of the IC_{50} for binding to a single potency site.
[b] Negative logarithm of the IC_{50} for the high-potency agonist binding site; percent of binding sites shown in parentheses.
[c] Negative logarithm of the IC_{50} for the low-potency agonist binding site.
[d] Maximal percent inhibition of radioligand binding.
[e] Control cells were incubated simultaneously with xanomeline and the radioligand.

~ Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of QNB binding between the 1min/wash/no wait group compared to control.

± ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of QNB binding where indicated between the 1min/wash/24h wait or 24h/wash/no wait pretreated groups compared to xanomeline 1min/wash/no wait treatment.

Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of QNB binding between the 1h/wash/no wait group compared to control.

† ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of QNB binding where indicated between the 1h/wash/23h wait or 24h/wash/no wait pretreated groups compared to xanomeline 1h/wash/no wait treatment.

& Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of NMS binding between the 1min/wash/no wait group compared to control.

@ ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of NMS binding where indicated between the 1min/wash/24h wait or 24h/wash/no wait groups compared to xanomeline 1min/wash/no wait treatment.

$ Unpaired t-test detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of NMS binding between the 1h/wash/no wait group compared to control.

* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in pIC_{50} of inhibition of NMS binding where indicated between the 1h/wash/23h wait or 24h/wash/no wait groups compared to xanomeline 1h/wash/no wait treatment.

‡ Significantly different ($p < 0.05$) from 100 percent as determined by ANOVA followed by Dunnett's post-test comparison.

Table 2. Effects of blocking receptor activation on xanomeline-induced changes in [^{3}H]NMS saturation binding parameters in CHO cells expressing hM3 receptors. Cells were treated with 10 μM xanomeline for 1 hour at 37ºC followed by washing and used after 23 hour incubation in control media at 4ºC. Additional cells were treated with 10 or 100 μM xanomeline for 24 hours at 4ºC prior to washing. Untreated control cells were incubated at 4ºC for 24 hours. Subsequently, untreated or treated cells were incubated with increasing concentrations of [^{3}H]NMS (0.03 – 16.6) at 37ºC for 1 hour. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

Xanomeline pretreatment group	K_d [a] (nM)	B_{max} [b]
Control (untreated) 4ºC	0.24 ± 0.004	35000 ± 2800
1 h 37ºC /washout/23 h 4ºC wait	0.34 ± 0.03	33400 ± 3600
24 h 4ºC (10 μM)/washout/no wait	1.08 ± 0.06*	35300 ± 4100
24 h 4ºC (100 μM)/washout/no wait	6.25 ± 0.44*	32500 ± 2000

[a] Equilibrium dissociation constant for [^{3}H]NMS binding.
[b] Cell-surface receptor density (dpm/well).
* ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in K_d or B_{max} where indicated between the pretreated groups compared with control.

Table 3. Effects of changing the level of receptor expression or inactivating the G proteins on xanomeline-induced changes in [^{3}H]NMS saturation binding parameters in CHO cells expressing the hM4 receptor subtype. Cells were pretreated with PrBCM (2 nM) for 15 minutes followed by washing and then subjected to xanomeline pretreatments. Additional cells were treated with pertussis toxin (100 ng/ml) for 24 hours prior to xanomeline pretreatments as noted in methods. Xanomeline treatment: cells were treated with 10 μM xanomeline for 1 hour followed by washing and incubation for 23 hours in the absence of free ligand. An additional group of cells was treated with xanomeline for 24 hours prior to washing. Control untreated or xanomeline pretreated cells were then incubated with increasing concentrations of [^{3}H]NMS (0.01 – 7.5 nM) for 1 hour at 37ºC. Parameters are from a single experiment.

	PrBCM		**Pertussis toxin**	
	K_d [a]	**B_{max}** [b]	**K_d**	**B_{max}**
Control (untreated)	0.44	93600	0.37	84900
1 h/washout/23h wait	0.28	34300	0.36	23600
24 h/washout/no wait	3.32	40200	1.26	12700

[a] Equilibrium dissociation constant for [^{3}H]NMS binding.
[b] Cell surface receptor density (dpm/well).
Statistical analyses were not performed because the data presented are from single experiments conducted in triplicate.

CHAPTER 7: Conclusions

Conclusions

Muscarinic receptors are potential targets for the development of drugs to treat diseases of the central nervous system such as Alzheimer's disease and schizophrenia. Currently, cholinergic drug treatment has shown promise in reducing symptoms such as cognitive deficits, hallucinations and delusions, which are thought to be mediated by the M1 and perhaps M4 muscarinic receptors. However, there are negative side-effects observed with cholinergic drugs that are thought to be mediated by non-selective activation of M2, M3 and M5 muscarinic receptor subtypes. A major goal of current research in muscarinic receptor biology and pharmacology is to develop agonists and antagonists that act in a subtype-selective manner, potentially through binding to allosteric sites on the receptor that are less conserved among subtypes. In order to accomplish this, we need to learn more about the allosteric site(s) on the muscarinic receptors and how potential drugs interact with these regions to modify the action of the receptor.

My thesis addressed a small part of this goal by focusing on the consequences of the long-term interaction of the novel muscarinic receptor agonist xanomeline with M1-M4 subtypes of muscarinic receptors, with a more detailed focus on the mechanisms of action of xanomeline at the M1 and M3 subtypes of muscarinic receptors. Xanomeline has been shown to act as an allosteric modulator of at least the M1 muscarinic receptor. More specifically, the questions that were asked were 1) does xanomeline bind to all receptor subtypes in a reversible and wash-resistant manner?, 2) what are the short- and long-term consequences of xanomeline binding on function at the M1 and M3 receptor subtypes?, 3) what is the role of the orthosteric binding site in the long-term effects of xanomeline at the M1 and M3 receptor subtypes? and 4) what is the

mechanism of receptor regulation as a result of the long-term consequences of xanomeline binding?

My research demonstrated that xanomeline is able to bind in a reversible manner at the orthosteric site and in a wash-resistant manner at an allosteric site at all of the subtypes tested. Interestingly, the potency of xanomeline binding across the four subtypes was similar when xanomeline was present acutely. However, differences in the binding profiles were apparent between subtypes when xanomeline was present for prolonged periods of time. These differences were most striking at the M2 receptor. However, there were also subtle differences between the other subtypes with the M1 and M3 receptors having the most similar binding profiles. My research further supports that xanomeline interacts with muscarinic receptors in a manner that is unique from other conventional muscarinic agonists and that there is a time-dependent component associated with that interaction. These results are important because it gives some insight into the consequences of prolonged binding of an agonist at the allosteric site.

The unique binding properties of xanomeline led us to determine the functional consequences of xanomeline exposure at the M1 and M3 receptor subtypes. Xanomeline was shown to be a full agonist at the M1 receptor and a partial agonist at the M3 receptor. Wash-resistant binding of xanomeline is able to stimulate a functional response at both receptor subtypes. Both acute and long-term treatments with xanomeline modulate the ability of additional agonists to stimulate a response. The major difference that is observed as a result of xanomeline treatment between the two subtypes is that the presence of xanomeline antagonizes the response to the subsequent addition of agonists to a greater extent at the M3 receptor than at the M1 receptor. These results demonstrate that the minor differences observed in binding studies are mirrored by minor differences in effects on receptor function. Furthermore,

these results are important because they demonstrate the ability of xanomeline to modulate the function of the receptor through binding at an allosteric site.

However, the long-term effects of xanomeline exposure are quite similar at the M1 and M3 muscarinic receptors in terms of cell-surface receptor density and radioligand binding affinity. In both cases activation of the orthosteric site is required to obtain the effects of long-term receptor regulation by xanomeline. This is of interest because xanomeline has been found to be a full agonist at the M1 receptor, but a partial agonist at the M3 receptor. This suggests that although there may be some degree of functional subtype selectivity initially, there is relatively little selectivity in the long-term effects of xanomeline exposure between these two subtypes. This is a potential problem for using xanomeline to selectively target M1 receptors. This is also important because it emphasizes that when drugs are being screened it is not only important to determine the properties of the initial exposure, but the consequences of prolonged exposure also need to be considered.

Furthermore, my research demonstrates that the mechanism of receptor regulation following prolonged exposure to xanomeline is most likely via down-regulation of the receptor. Again, xanomeline exposure appears to result in receptor modulation that is similar between the M1 and M3 receptor subtypes, although the time dependence of this is slightly different. These results are similar to what is observed following prolonged exposure of muscarinic receptors to conventional muscarinic receptor agonists, further supporting the role of the orthosteric binding site in the long-term effects of xanomeline. Interestingly, it appears that the mechanism of receptor regulation is different between the M2 and M4 receptor subtypes compared to the M1 and M3 receptor subtypes. Blocking G protein function at the M4 receptor had no effect on the long-term consequences of xanomeline exposure; however, blocking signaling through

the G protein at the M1 and M3 receptors abolished the long-term effects of xanomeline. These results demonstrate that the nature of drug-receptor interactions is not only dependent upon the type of binding, but also the signaling mechanisms that are activated subsequent to binding.

All of the experiments that I presented were conducted in an *in vitro* receptor expression system; individual receptors expressed in CHO cells. This approach has its strengths and drawbacks. The advantage of using this type of experimental set up is that only a single subtype of receptor is expressed so the actions of xanomeline at a particular subtype can be determined. This would not be possible using tissue preparations since tissues are known to express multiple subtypes of muscarinic receptors. However, there are potential problems associated with the use of receptor expression systems such as there may be differences in the coupling of G proteins and other associated proteins than would be observed endogenously, as well as receptor overexpression could result in changes in binding and functional properties of xanomeline. Finally, it is possible that the concentrations of xanomeline tested in these experiments may be higher than the concentrations that would be found at the target site(s) *in vivo*. Thus, further experimentation *in vivo* is necessary to confirm the results that we obtained in our system and further elucidate the effects of xanomeline treatment.

In addition to conducting further experiments *in vivo,* there are other questions that still remain that would be more easily addressed *in vitro*. Some of these questions include, Where is the allosteric site that xanomeline binds to located? Is the allosteric site located in similar regions across receptor subtypes? What residues are important in the development of wash-resistant binding? Is the allosteric site unique to xanomeline or do other allosteric modulators utilize the same site? What is the signaling pathway/mechanism that is activated as a result of xanomeline stimulation? Are there

any interactions/what are the interactions between receptor subtypes when exposed to xanomeline? These questions can be addressed using a variety of different methods. For example, studies utilizing receptor mutagenesis can be employed to determine the location of the allosteric binding pocket and the residues important in the formation of wash-resistant binding. The creation of receptor chimeras could address questions about the signaling pathways that are involved. Furthermore, expression of multiple subtypes of receptors in the same cell would be useful in determining the potential interaction between different subtypes of muscarinic receptors following drug exposure. It would also be important to extend these studies to look at other allosteric modulators of muscarinic receptors to determine if there is a common mechanism of action between allosteric modulators or if each modulator acts in its own specific manner. Together the answers to these questions would give us more insight and understanding of the mechanisms of action of allosteric modulators of muscarinic receptors. This information would aid in the development of agonists and antagonists that act in a subtype selective manner.

In summary, my data demonstrate that the novel muscarinic receptor agonist xanomeline interacts with muscarinic receptors in a unique manner. Although the acute effects of xanomeline exposure are similar across subtypes the long-term effects are somewhat distinctive between the receptor subtypes. The binding and functional characteristics of xanomeline confirm that it is unlikely to be a beneficial therapeutic due to its interaction with multiple muscarinic receptor subtypes. Thus, my research demonstrates that it is important to determine the long-term interactions of drugs on receptor properties in addition to the acute effects as part of the drug discovery and development process. This is an exciting time for the field of muscarinic receptor biology. A plethora of new research is directed at understanding and developing

allosteric modulators of muscarinic receptors in the hope that they will display subtype selectivity and thus can be used in the treatment of various diseases of the central nervous system.

References

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM,Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nat Neurosci. 6: 51-58.

Andersen MB, Fink-Jensen A, Peacock L, Gerlach J, Bymaster F, Lundbaek JA,Werge T (2003) The muscarinic M1/M4 receptor agonist xanomeline exhibits antipsychotic-like activity in Cebus apella monkeys. Neuropsychopharmacology. 28: 1168-1175.

Ashe KH (2005) Mechanisms of memory loss in Abeta and tau mouse models. Biochem Soc Trans. 33: 591-594.

Ben-Barak J,Dudai Y (1980) Scopolamine induces an increase in muscarinic receptor level in rat hippocampus. Brain Res. 193: 309-313.

Berridge MJ, Dawson RM, Downes CP, Heslop JP,Irvine RF (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J. 212: 473-482.

Bianchetti A, Ranieri P, Margiotta A,Trabucchi M (2006) Pharmacological treatment of Alzheimer's Disease. Aging Clin Exp Res. 18: 158-162.

Birdsall NJ,Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. Mini Rev Med Chem. 5: 523-543.

Birdsall NJ, Lazareno S,Matsui H (1996) Allosteric regulation of muscarinic receptors. Prog Brain Res. 109: 147-151.

Blennow K (2004) Cerebrospinal fluid protein biomarkers for Alzheimer's disease. NeuroRx. 1: 213-225.

Bodick NC, Offen WW, Shannon HE, Satterwhite J, Lucas R, van Lier R,Paul SM (1997 (b)) The selective muscarinic agonist xanomeline improves both the cognitive deficits and behavioral symptoms of Alzheimer disease. Alzheimer Dis Assoc Disord. 11 Suppl 4: S16-22.

Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A, Shannon HE, Tollefson GD, Rasmussen K, Bymaster FP, Hurley DJ, Potter WZ,Paul SM (1997 (a)) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. Arch Neurol. 54: 465-473.

Bonner TI (1989) The molecular basis of muscarinic receptor diversity. Trends Neurosci. 12: 148-151.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254.

Bymaster FP, Felder C, Ahmed S,McKinzie D (2002) Muscarinic receptors as a target for drugs treating schizophrenia. Curr Drug Targets CNS Neurol Disord. 1: 163-181.

Bymaster FP, Wong DT, Mitch CH, Ward JS, Calligaro DO, Schoepp DD, Shannon HE, Sheardown MJ, Olesen PH, Suzdak PD,et al. (1994) Neurochemical effects of the M1 muscarinic agonist xanomeline (LY246708/NNC11-0232). J Pharmacol Exp Ther. 269: 282-289.

Bymaster FP, Whitesitt CA, Shannon HE, DeLapp N, Ward JS, Calligaro DO, Shipley LA, Buelke-Sam JL, Bodick NC, Sheardown FL, Olesen PH, Hansen KT, Suzdak PD, Swedberg MDB, Sauerberg P,Mitch CH (1997) Xanomeline: A selective muscarinic agonist for the treatment of Alzhiemer's disease. Drug Development Research. 40: 158-170.

Campbell A (2004) Inflammation, neurodegenerative diseases, and environmental exposures. Ann N Y Acad Sci. 1035: 117-132.

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

Caulfield MP,Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev. 50: 279-290.

Chan (a) AL, Chien YW,Jin Lin S (2008) Transdermal delivery of treatment for Alzheimer's disease: development, clinical performance and future prospects. Drugs Aging. 25: 761-775.

Chen G, Way J, Armour S, Watson C, Queen K, Jayawickreme CK, Chen WJ,Kenakin T (2000) Use of constitutive G protein-coupled receptor activity for drug discovery. Mol Pharmacol. 57: 125-134.

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. Nat Rev Drug Discov. 1: 198-210.

Christopoulos A,El-Fakahany EE (1997) Novel persistent activation of muscarinic M1 receptors by xanomeline. Eur J Pharmacol. 334: R3-4.

Christopoulos A,El-Fakahany EE (1999) Qualitative and quantitative assessment of relative agonist efficacy. Biochem Pharmacol. 58: 735-748.

Christopoulos A,Kenakin T (2002) G protein-coupled receptor allosterism and complexing. Pharmacol Rev. 54: 323-374.

Christopoulos A, Parsons AM,El-Fakahany EE (1999) Pharmacological analysis of the novel mode of interaction between xanomeline and the M1 muscarinic acetylcholine receptor. J Pharmacol Exp Ther. 289: 1220-1228.

Christopoulos A, Pierce TL, Sorman JL,El-Fakahany EE (1998) On the unique binding and activating properties of xanomeline at the M1 muscarinic acetylcholine receptor. Mol Pharmacol. 53: 1120-1130.

Christopoulos A, Sorman JL, Mitchelson F,El-Fakahany EE (1999) Characterization of the subtype selectivity of the allosteric modulator heptane-1,7-bis-(dimethyl-3'-phthalimidopropyl) ammonium bromide (C7/3-phth) at cloned muscarinic acetylcholine receptors. Biochem Pharmacol. 57: 171-179.

Cioffi CL,El-Fakahany EE (1989) Differential sensitivity of phosphoinositide and cyclic GMP responses to short-term regulation by a muscarinic agonist in mouse neuroblastoma cells. Correlation with down-regulation of cell surface receptors. Biochem Pharmacol. 38: 1827-1834.

Clark AL,Mitchelson F (1976) The inhibitory effect of gallamine on muscarinic receptors. Br J Pharmacol. 58: 323-331.

Coleman RA, Johnson M, Nials AT,Vardey CJ (1996) Exosites: their current status, and their relevance to the duration of action of long-acting beta 2-adrenoceptor agonists. Trends Pharmacol Sci. 17: 324-330.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL,Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 261: 921-923.

Crespo P, Xu N, Simonds WF,Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature. 369: 418-420.

De Lorme KC, Sikorski KL, Grant MK,El-Fakahany EE (2006) Long-term wash-resistant effects of brief interaction of xanomeline at the M1 muscarinic receptor. Neurosci Lett. 410: 11-14.

De Lorme KC, Grant MK, Noetzel MJ, Polson SB,El-Fakahany EE (2007) Long-term changes in the muscarinic M1 receptor induced by instantaneous formation of wash-resistant xanomeline-receptor complex. J Pharmacol Exp Ther. 323: 868-876.

Disterhoft JF,Oh MM (2006) Pharmacological and molecular enhancement of learning in aging and Alzheimer's disease. J Physiol Paris. 99: 180-192.

Dohlman HG, Thorner J, Caron MG,Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem. 60: 653-688.

Eglen RM (2005) Muscarinic receptor subtype pharmacology and physiology. Prog Med Chem. 43: 105-136.

Eglen RM, Huff MM, Montgomery WW,Whiting RL (1987) Differential effects of pertussis toxin and lithium on muscarinic responses in the atria and ileum: evidence for receptor heterogeneity. Br J Pharmacol. 91: 6-8.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. Mol Pharmacol. 33: 187-194.

Ehlert FJ, Roeske WR,Yamamura HI (1981) Muscarinic receptor: regulation by guanine nucleotides, ions, and N-ethylmaleimide. Fed Proc. 40: 153-159.

El-Fakahany E,Richelson E (1980) Temperature dependence of muscarinic acetylcholine receptor activation, desensitization, and resensitization. J Neurochem. 34: 1288-1295.

El-Fakahany EE,Cioffi CL (1990) Molecular mechanisms of regulation of neuronal muscarinic receptor sensitivity. Membr Biochem. 9: 9-27.

Feigenbaum P,El-Fakahany EE (1984) Short-term regulation of muscarinic acetylcholine receptor binding in cultured nerve cells. Res Commun Chem Pathol Pharmacol. 43: 519-522.

Feigenbaum P,El-Fakahany EE (1985) Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist: possible involvement in desensitization of receptor function. J Pharmacol Exp Ther. 233: 134-140.

Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. Faseb J. 9: 619-625.

Fisher A, Heldman E, Gurwitz D, Haring R, Karton Y, Meshulam H, Pittel Z, Marciano D, Brandeis R, Sadot E, Barg Y, Pinkas-Kramarski R, Vogel Z, Ginzburg I, Treves TA, Verchovsky R, Klimowsky S,Korczyn AD (1996) M1 agonists for the treatment of Alzheimer's disease. Novel properties and clinical update. Ann N Y Acad Sci. 777: 189-196.

Flynn DD, Weinstein DA,Mash DC (1991) Loss of high-affinity agonist binding to M1 muscarinic receptors in Alzheimer's disease: implications for the failure of cholinergic replacement therapies. Ann Neurol. 29: 256-262.

Flynn DD, Ferrari-DiLeo G, Levey AI,Mash DC (1995) Differential alterations in muscarinic receptor subtypes in Alzheimer's disease: implications for cholinergic-based therapies. Life Sci. 56: 869-876.

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

Friedman JI (2004) Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. Psychopharmacology (Berl). 174: 45-53.

Fukamauchi F, Saunders PA, Hough C,Chuang DM (1993) Agonist-induced down-regulation and antagonist-induced up-regulation of m2- and m3-muscarinic acetylcholine receptor mRNA and protein in cultured cerebellar granule cells. Mol Pharmacol. 44: 940-949.

Gao ZG,Jacobson KA (2006) Keynote review: allosterism in membrane receptors. Drug Discov Today. 11: 191-202.

Gautam D, Gavrilova O, Jeon J, Pack S, Jou W, Cui Y, Li JH,Wess J (2006) Beneficial metabolic effects of M3 muscarinic acetylcholine receptor deficiency. Cell Metab. 4: 363-375.

Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev. 21: 90-113.

Grant MK,El-Fakahany EE (2005) Persistent binding and functional antagonism by xanomeline at the muscarinic M5 receptor. J Pharmacol Exp Ther. 315: 313-319.

Grimes ML,Miettinen HM (2003) Receptor tyrosine kinase and G-protein coupled receptor signaling and sorting within endosomes. J Neurochem. 84: 905-918.

Han SJ, Hamdan FF, Kim SK, Jacobson KA, Bloodworth LM, Li B,Wess J (2005) Identification of an agonist-induced conformational change occurring adjacent to the ligand-binding pocket of the M(3) muscarinic acetylcholine receptor. J Biol Chem. 280: 34849-34858.

Hardy J, Duff K, Hardy KG, Perez-Tur J,Hutton M (1998) Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. Nat Neurosci. 1: 355-358.

Hodes RJ (2006) Public funding for Alzheimer disease research in the United States. Nat Med. 12: 770-773.

Holroyd EW, Szekeres PG, Whittaker RD, Kelly E,Edwardson JM (1999) Effect of G protein-coupled receptor kinase 2 on the sensitivity of M4 muscarinic acetylcholine receptors to agonist-induced internalization and desensitization in NG108-15 cells. J Neurochem. 73: 1236-1245.

Hu J, Wang SZ,El-Fakahany EE (1991) 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: 938-945.

Hu JR,El-Fakahany EE (1990) Selectivity of McN-A-343 in stimulating phosphoinositide hydrolysis mediated by M1 muscarinic receptors. Mol Pharmacol. 38: 895-903.

Hulme EC, Lu ZL,Bee MS (2003) Scanning mutagenesis studies of the M1 muscarinic acetylcholine receptor. Receptors Channels. 9: 215-228.

Jakubik J, Tucek S,El-Fakahany EE (2002) Allosteric modulation by persistent binding of xanomeline of the interaction of competitive ligands with the M1 muscarinic acetylcholine receptor. J Pharmacol Exp Ther. 301: 1033-1041.

Jakubik J, Tucek S,El-Fakahany EE (2004) Role of receptor protein and membrane lipids in xanomeline wash-resistant binding to muscarinic M1 receptors. J Pharmacol Exp Ther. 308: 105-110.

Jakubik J, El-Fakahany EE,Dolezal V (2006) Differences in kinetics of xanomeline binding and selectivity of activation of G proteins at M(1) and M(2) muscarinic acetylcholine receptors. Mol Pharmacol. 70: 656-666.

Jakubik J, Bacakova L, El-Fakahany EE,Tucek S (1997) Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. Mol Pharmacol. 52: 172-179.

Jones CK, Eberle EL, Shaw DB, McKinzie DL,Shannon HE (2005) Pharmacologic interactions between the muscarinic cholinergic and dopaminergic systems in the modulation of prepulse inhibition in rats. J Pharmacol Exp Ther. 312: 1055-1063.

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW,Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci. 28: 10422-10433.

Kane BE, Grant MK, El-Fakahany EE,Ferguson DM (2008) Synthesis and evaluation of xanomeline analogs--probing the wash-resistant phenomenon at the M1 muscarinic acetylcholine receptor. Bioorg Med Chem. 16: 1376-1392.

Keller JN (2006) Age-related neuropathology, cognitive decline, and Alzheimer's disease. Ageing Res Rev. 5: 1-13.

Kenakin T (1997) Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. Trends Pharmacol Sci. 18: 456-464.

Kenakin T (2003) Ligand-selective receptor conformations revisited: the promise and the problem. Trends Pharmacol Sci. 24: 346-354.

Kotilinek LA, Bacskai B, Westerman M, Kawarabayashi T, Younkin L, Hyman BT, Younkin S,Ashe KH (2002) Reversible memory loss in a mouse transgenic model of Alzheimer's disease. J Neurosci. 22: 6331-6335.

Lanzafame AA, Christopoulos A,Mitchelson F (2003) Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels. 9: 241-260.

Lanzafame AA, Sexton PM,Christopoulos A (2006) Interaction studies of multiple binding sites on m4 muscarinic acetylcholine receptors. Mol Pharmacol. 70: 736-746.

Lee NH,El-Fakahany EE (1991) Allosteric antagonists of the muscarinic acetylcholine receptor. Biochem Pharmacol. 42: 199-205.

Lee W,Wolfe BB (1989) Regulation of muscarinic receptor subtypes and their responsiveness in rat brain following chronic atropine administration. Mol Pharmacol. 36: 749-757.

Lefkowitz RJ,Caron MG (1988) Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. J Biol Chem. 263: 4993-4996.

Lefkowitz RJ, Cotecchia S, Samama P,Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. Trends Pharmacol Sci. 14: 303-307.

Maccioni RB, Lavados M, Maccioni CB,Mendoza-Naranjo A (2004) Biological markers of Alzheimer's disease and mild cognitive impairment. Curr Alzheimer Res. 1: 307-314.

Machova E, Jakubik J, El-Fakahany EE,Dolezal V (2007) Wash-resistantly bound xanomeline inhibits acetylcholine release by persistent activation of presynaptic M(2) and M(4) muscarinic receptors in rat brain. J Pharmacol Exp Ther. 322: 316-323.

Maloteaux JM,Hermans E (1994) Agonist-induced muscarinic cholinergic receptor internalization, recycling and degradation in cultured neuronal cells. Cellular mechanisms and role in desensitization. Biochem Pharmacol. 47: 77-88.

Maloteaux JM, Gossuin A, Pauwels PJ,Laduron PM (1983) Short-term disappearance of muscarinic cell surface receptors in carbachol-induced desensitization. FEBS Lett. 156: 103-107.

May LT, Lin Y, Sexton PM,Christopoulos A (2005) Regulation of M2 muscarinic acetylcholine receptor expression and signaling by prolonged exposure to allosteric modulators. J Pharmacol Exp Ther. 312: 382-390.

Messer WS, Jr. (2002) Cholinergic agonists and the treatment of Alzheimer's disease. Curr Top Med Chem. 2: 353-358.

Mesulam MM (2004) The cholinergic innervation of the human cerebral cortex. Prog Brain Res. 145: 67-78.

Milligan G,Strange PG (1983) Apparent down-regulation of muscarinic acetylcholine receptors of neuroblastoma cells by pilocarpine is due to occluded agonist. Eur J Pharmacol. 91: 223-228.

Milligan G, Bond RA,Lee M (1995) Inverse agonism: pharmacological curiosity or potential therapeutic strategy? Trends Pharmacol Sci. 16: 10-13.

Minghetti L (2005) Role of inflammation in neurodegenerative diseases. Curr Opin Neurol. 18: 315-321.

Mirza NR, Peters D,Sparks RG (2003) Xanomeline and the antipsychotic potential of muscarinic receptor subtype selective agonists. CNS Drug Rev. 9: 159-186.

Mistry R, Dowling MR,Challiss RA (2005) An investigation of whether agonist-selective receptor conformations occur with respect to M2 and M4 muscarinic acetylcholine receptor signalling via Gi/o and Gs proteins. Br J Pharmacol. 144: 566-575.

Mueser KT,McGurk SR (2004) Schizophrenia. Lancet. 363: 2063-2072.

Mullaney I,Milligan G (1993) Agonist activation of transfected human M1 muscarinic acetylcholine receptor in Chinese hamster ovary cells results in concurrent downregulation of Gq alpha and G11 alpha. Biochem Soc Trans. 21: 497S.

Nahorski SR, Tobin AB,Willars GB (1997) Muscarinic M3 receptor coupling and regulation. Life Sci. 60: 1039-1045.

Nathanson NM (2000) A multiplicity of muscarinic mechanisms: enough signaling pathways to take your breath away. Proc Natl Acad Sci U S A. 97: 6245-6247.

Nelson CP, Nahorski SR,Challiss RA (2006) Constitutive activity and inverse agonism at the M2 muscarinic acetylcholine receptor. J Pharmacol Exp Ther. 316: 279-288.

Nials AT, Sumner MJ, Johnson M,Coleman RA (1993) Investigations into factors determining the duration of action of the beta 2-adrenoceptor agonist, salmeterol. Br J Pharmacol. 108: 507-515.

Oda Y (1999) Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. Pathol Int. 49: 921-937.

Pitcher JA, Freedman NJ,Lefkowitz RJ (1998) G protein-coupled receptor kinases. Annu Rev Biochem. 67: 653-692.

Raedler TJ, Bymaster FP, Tandon R, Copolov D,Dean B (2007) Towards a muscarinic hypothesis of schizophrenia. Mol Psychiatry. 12: 232-246.

Reddy PH (2006) Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. J Neurochem. 96: 1-13.

Shannon HE, Bymaster FP, Calligaro DO, Greenwood B, Mitch CH, Sawyer BD, Ward JS, Wong DT, Olesen PH, Sheardown MJ,et al. (1994) Xanomeline: a novel muscarinic receptor agonist with functional selectivity for M1 receptors. J Pharmacol Exp Ther. 269: 271-281.

Shekhar A, Potter WZ, Lightfoot J, Lienemann J, Dube S, Mallinckrodt C, Bymaster FP, McKinzie DL,Felder CC (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry. 165: 1033-1039.

Shifrin GS,Klein WL (1980) Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. J Neurochem. 34: 993-999.

Shirey JK, Xiang Z, Orton D, Brady AE, Johnson KA, Williams R, Ayala JE, Rodriguez AL, Wess J, Weaver D, Niswender CM,Conn PJ (2008) An allosteric potentiator of M4 mAChR modulates hippocampal synaptic transmission. Nat Chem Biol. 4: 42-50.

Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U,Brann MR (2002) Discovery of an ectopic activation site on the M(1) muscarinic receptor. Mol Pharmacol. 61: 1297-1302.

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR,Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and N-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol. 70: 1974-1983.

Sramek JJ, Hurley DJ, Wardle TS, Satterwhite JH, Hourani J, Dies F,Cutler NR (1995) The safety and tolerance of xanomeline tartrate in patients with Alzheimer's disease. J Clin Pharmacol. 35: 800-806.

Stanhope KJ, Mirza NR, Bickerdike MJ, Bright JL, Harrington NR, Hesselink MB, Kennett GA, Lightowler S, Sheardown MJ, Syed R, Upton RL, Wadsworth G, Weiss SM,Wyatt A (2001) The muscarinic receptor agonist xanomeline has an antipsychotic-like profile in the rat. J Pharmacol Exp Ther. 299: 782-792.

Steel MC,Buckley NJ (1993) Differential regulation of muscarinic receptor mRNA levels in neuroblastoma cells by chronic agonist exposure: a comparative polymerase chain reaction study. Mol Pharmacol. 43: 694-701.

Stope MB, Kunkel C, Kories C, Schmidt M,Michel MC (2003) Differential agonist-induced regulation of human M2 and M3 muscarinic receptors. Biochem Pharmacol. 66: 2099-2105.

Suzuki Y, Yamamoto S, Umegaki H, Onishi J, Mogi N, Fujishiro H,Iguchi A (2004) Smell identification test as an indicator for cognitive impairment in Alzheimer's disease. Int J Geriatr Psychiatry. 19: 727-733.

Tejada FR, Nagy PI, Xu M, Wu C, Katz T, Dorsey J, Rieman M, Lawlor E, Warrier M,Messer WS, Jr. (2006) Design and synthesis of novel derivatives of the muscarinic agonist tetra(ethylene glycol)(3-methoxy-1,2,5-thiadiazol-4-yl) [3-(1-methyl-1,2,5,6-tetrahydropyrid-3-yl)-1,2,5-thiadiazol-4-yl] ether (CDD-0304): effects of structural modifications on the binding and activity at muscarinic receptor subtypes and chimeras. J Med Chem. 49: 7518-7531.

Thompson AK,Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. J Pharmacol Exp Ther. 252: 744-752.

Thor D, Schulz A, Hermsdorf T,Schoneberg T (2008) Generation of an agonistic binding site for blockers of the M(3) muscarinic acetylcholine receptor. Biochem J. 412: 103-112.

Tsuga H, Kameyama K,Haga T (1998) Desensitization of human muscarinic acetylcholine receptor m2 subtypes is caused by their sequestration/internalization. J Biochem. 124: 863-868.

Tucek S,Proska J (1995) Allosteric modulation of muscarinic acetylcholine receptors. Trends Pharmacol Sci. 16: 205-212.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M,Mailman RB (2007)

Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther. 320: 1-13.

van de Westerlo E, Yang J, Logsdon C,Williams JA (1995) Down-regulation of the G-proteins Gq alpha and G11 alpha by transfected human M3 muscarinic acetylcholine receptors in Chinese hamster ovary cells is independent of receptor down-regulation. Biochem J. 310 (Pt 2): 559-563.

van Koppen CJ,Kaiser B (2003) Regulation of muscarinic acetylcholine receptor signaling. Pharmacol Ther. 98: 197-220.

Vickroy TW,Malphurs WL (1994) Irreversible blockade of high-affinity choline uptake in rat brain by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). Biochem Pharmacol. 48: 1281-1287.

Volpicelli LA,Levey AI (2004) Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus. Prog Brain Res. 145: 59-66.

Wang SZ,El-Fakahany EE (1993) Application of transfected cell lines in studies of functional receptor subtype selectivity of muscarinic agonists. J Pharmacol Exp Ther. 266: 237-243.

Wang SZ, Zhu SZ,el-Fakahany EE (1995) Expression of endogenous muscarinic acetylcholine receptors in Chinese hamster ovary cells. Eur J Pharmacol. 291: R1-2.

Wang SZ, Hu JR, Long RM, Pou WS, Forray C,el-Fakahany EE (1990) Agonist-induced down-regulation of m1 muscarinic receptors and reduction of their mRNA level in a transfected cell line. FEBS Lett. 276: 185-188.

Ward SD, Hamdan FF, Bloodworth LM,Wess J (2002) Conformational changes that occur during M3 muscarinic acetylcholine receptor activation probed by the use of an in situ disulfide cross-linking strategy. J Biol Chem. 277: 2247-2257.

Werry TD, Sexton PM,Christopoulos A (2005) "Ins and outs" of seven-transmembrane receptor signalling to ERK. Trends Endocrinol Metab. 16: 26-33.

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol. 10: 69-99.

Wess J (2003) Novel insights into muscarinic acetylcholine receptor function using gene targeting technology. Trends Pharmacol Sci. 24: 414-420.

Wess J (2004) Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu Rev Pharmacol Toxicol. 44: 423-450.

Wess J (2005) Allosteric binding sites on muscarinic acetylcholine receptors. Mol Pharmacol. 68: 1506-1509.

Wess J, Duttaroy A, Zhang W, Gomeza J, Cui Y, Miyakawa T, Bymaster FP, McKinzie L, Felder CC, Lamping KG, Faraci FM, Deng C,Yamada M (2003) M1-M5 muscarinic

receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system. Receptors Channels. 9: 279-290.

Winitz S, Russell M, Qian NX, Gardner A, Dwyer L,Johnson GL (1993) Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. J Biol Chem. 268: 19196-19199.

Wisniewski T,Konietzko U (2008) Amyloid-beta immunisation for Alzheimer's disease. Lancet Neurol. 7: 805-811.

Wood MD, Murkitt KL, Ho M, Watson JM, Brown F, Hunter AJ,Middlemiss DN (1999) Functional comparison of muscarinic partial agonists at muscarinic receptor subtypes hM1, hM2, hM3, hM4 and hM5 using microphysiometry. Br J Pharmacol. 126: 1620-1624.

Wotta DR, Wattenberg EV, Langason RB,el-Fakahany EE (1998) M1, M3 and M5 muscarinic receptors stimulate mitogen-activated protein kinase. Pharmacology. 56: 175-186.

Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, Felder CC, Deng CX,Wess J (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. Nature. 410: 207-212.

Yan Z,Feng J (2004) Alzheimer's disease: interactions between cholinergic functions and beta-amyloid. Curr Alzheimer Res. 1: 241-248.

Yang J, Logsdon CD, Johansen TE,Williams JA (1993) Human m3 muscarinic acetylcholine receptor carboxyl-terminal threonine resides are required for agonist-induced receptor down-regulation. Mol Pharmacol. 44: 1158-1164.

Zhu SZ, Wang SZ, Abdallah EA,el-Fakahany EE (1991) DFP-induced regulation of cardiac muscarinic receptor mRNA in vivo measured by DNA-excess solution hybridization. Life Sci. 48: 2579-2584.

Zimmermann M, Gardoni F,Di Luca M (2005) Molecular rationale for the pharmacological treatment of Alzheimer's disease. Drugs Aging. 22 Suppl 1: 27-37.

APPENDIX A: Differences observed between suspension and monolayer protocols

Introduction

The results of an experiment can be dependent on the protocol used to address the question. For example, previous research by Thompson & Fisher (1990) found that treating cells in monolayer with agonists resulted in a larger decrease in receptor expression levels compared to the treatment of cells in suspension. In another case, when forskolin-stimulated cAMP accumulation was studied in cells in suspension it only initiated 75 percent of the response that was observed in cells assayed in monolayer (Mistry et al., 2005). However, the opposite was true in another assay conducted by Mistry et al. (2005). In that case they found that pilocarpine elicited a larger response when cells were stimulated in suspension rather than in monolayer. Together, these results demonstrate that the response to agonists is dependent on both the assay conditions and the agonist being studied.

In our experimental protocols we noted some differences following xanomeline treatment depending on whether the assay was conducted on cells in monolayer or in suspension. To address this question we performed experiments utilizing various binding paradigms and compared the results between the protocols. Our data demonstrate that there are some differences between monolayer and suspension binding protocols following xanomeline treatments, so when possible experiments were conducted on cells in monolayer at all receptor subtypes.

Results

Comparison between saturation binding with xanomeline pretreatments conducted in monolayer versus in suspension in hM3 CHO cells

The binding profile of xanomeline was determined using two different protocols. In either case, pretreatment with 10 μM xanomeline for one hour was conducted on cells

in monolayer. Subsequently, cells were exposed to increasing concentrations of [^{3}H]NMS either in suspension or monolayer. Pretreatment with xanomeline for one hour followed by washing and immediate use in the suspension binding assay resulted in no change in cell-surface receptor density but a slight decrease in radioligand affinity compared to control; however, it did not reach significance (Fig. 1 a). In contrast, as presented in chapter 3, when the same pretreatment conditions were used but the assay was conducted in monolayer there was a significant ($p < 0.05$) decrease in radioligand affinity (Fig. 1 b). When cells were treated with xanomeline for one hour followed by washing and waiting 23 hours there was no change in radioligand affinity and only a slight decrease in cell-surface receptor density for cells that were assayed in suspension (Fig. 1 a). In contrast, the same pretreatments followed by assaying in monolayer resulted in a significant ($p < 0.05$) decrease in cell-surface receptor expression (Fig. 1 b). When pretreatment time with xanomeline was increased to 24 hours prior to washing and performing the binding assay in suspension there was a significant ($p < 0.05$) decrease in radioligand affinity, however, there was only a slight decrease in cell-surface receptor density (Fig. 1 a). In contrast, when the assay was conducted in monolayer there was a significant ($p < 0.05$) decrease in both parameters (Fig. 1 b). Results are summarized in Table 1.

Comparison between the shift in inhibition of [^{3}H]NMS binding curves following acute xanomeline exposure in monolayer versus in suspension at the hM1 CHO cells

Experiments were conducted to determine the inhibition of [^{3}H]NMS binding as a result of exposure of cells expressing the M1 receptor to xanomeline for one hour followed by washing and immediate use in the binding assay. These results were

compared to the ability of xanomeline to inhibit [^{3}H]NMS binding when it was present during the binding assay. When cells were assayed in suspension there was a decrease in potency of 1.8 orders of magnitude when xanomeline was present for one hour followed by washing (pEC_{50} 5.6) compared to when xanomeline was continually present (control; pEC_{50} 7.4) in the binding assay (Fig. 2 a). In contrast, when cells were assayed in monolayer the decrease in potency was only 0.5 orders of magnitude for xanomeline pretreatment (pEC_{50} 5.7) compared to control (pEC_{50} 6.2) (Fig. 2 b).

Discussion

Alterations in experimental protocols have previously been shown to result in changes in the binding and functional profiles of receptors. These changes appear to be a result of both the protocol employed and the agonist studied. Experiments were therefore conducted to determine if there were differences in the binding properties following exposure to xanomeline when cells were assayed in monolayer compared to suspension. Our results show that pretreatment with xanomeline resulted in a larger decrease in cell-surface receptor expression when cells expressing the M3 receptor were assayed in monolayer compared to suspension (Fig. 1). Additionally, the shift in potency between control and xanomeline treatment for one hour followed by washing and immediate use was larger for cells expressing the M1 receptor assayed in suspension compared to monolayer (Fig. 2).

The cause of the difference in binding profiles between the different assays was not studied in detail. However, a potential explanation could be that the differences are a result of differential effects of cytoskeletal elements in cells that are floating (suspension) versus attached (monolayer). Receptor activation has been shown to result in changes in cytoskeletal arrangement (Grimes & Miettinen, 2003). Furthermore,

endocytosis is thought to be dependent on recruitment of beta-arrestin, which is a scaffolding protein (Grimes & Miettinen, 2003). Additionally, it is possible that the procedure employed to detach the cells prior to the binding assay alters the binding profile of xanomeline that is bound in a wash-resistant manner.

Interestingly, the levels of protein for the saturation experiments with cells assayed in suspension were one tenth of the protein levels seen in monolayer. This difference corresponds to the ten fold difference in maximal cell-surface receptor expression levels observed between suspension and monolayer. Thus, the difference in saturation binding profiles between the two methods is most likely not a result of differences in receptor expression.

In summary, we addressed the question, is there a difference in the binding properties following exposure to xanomeline at either or both the M1 and M3 receptors when cells are assayed in suspension compared to monolayer? In both cases we observed slight differences in the binding properties suggesting that the difference is dependent on the protocol and not the specific receptor subtype studied. However, this was by no means an extensive study of the differences between the binding properties of cells assayed in monolayer compared to suspension, but rather an observation that was made early on that directed the protocols employed in further studies. Thus, when possible experimental assays were performed on cells in monolayer at all receptor subtypes so direct comparisons between groups could be made.

Figure 1

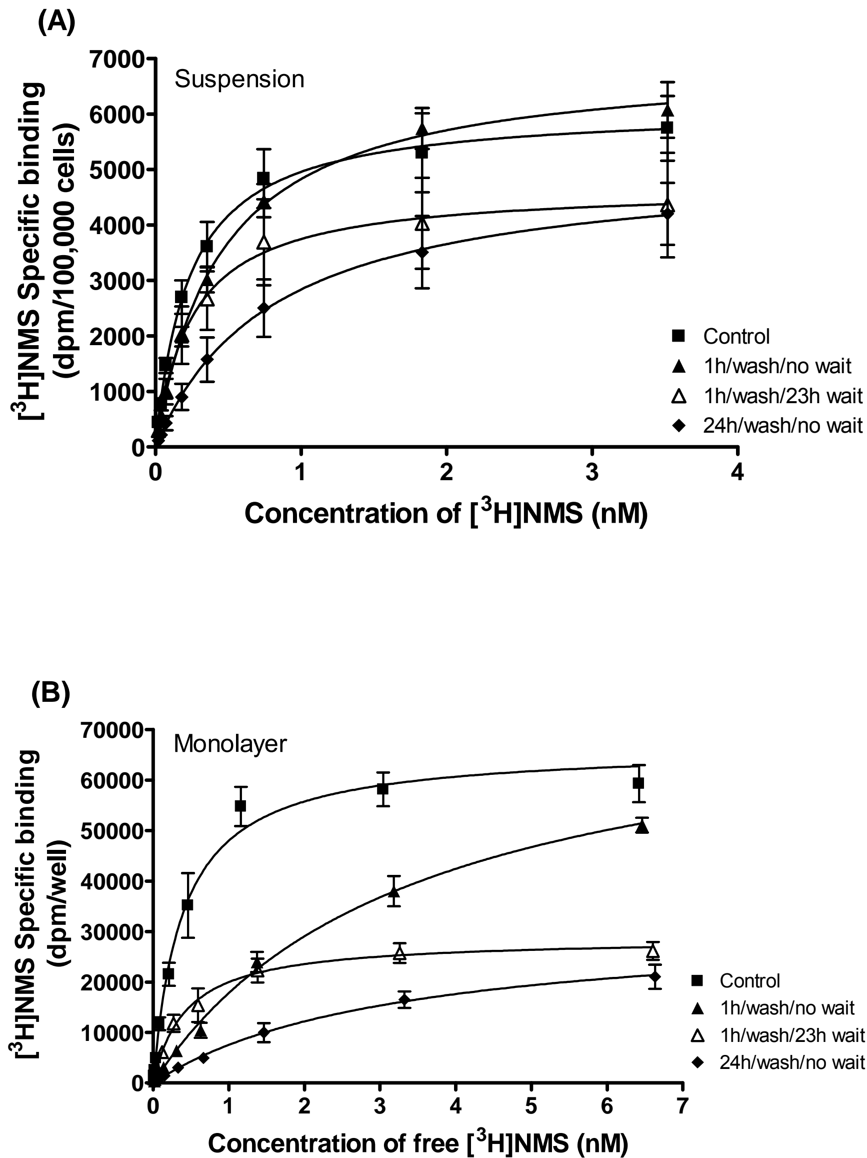


Figure 1. Comparison between [^{3}H]NMS saturation binding paradigms on assays conducted in suspension versus in monolayer in CHO cells stably expressing hM3 receptors. Cells were pretreated with 10 µM xanomeline for 1 hour followed by washing and used immediately (closed triangles) or subsequent to 23 hour incubation in ligand free media (open triangles). Additional cells were treated with xanomeline for 24 hours prior to washing (closed diamonds). Subsequently, untreated (closed squares) or xanomeline pretreated cells were incubated with increasing concentrations of [^{3}H]NMS (0.01 –6.6 nM) for 1 hour at 37°C. The radioligand binding assay was conducted in A) suspension or B) monolayer. Values represent the means ± S.E. of 4 experiments conducted in triplicate.

Figure 2

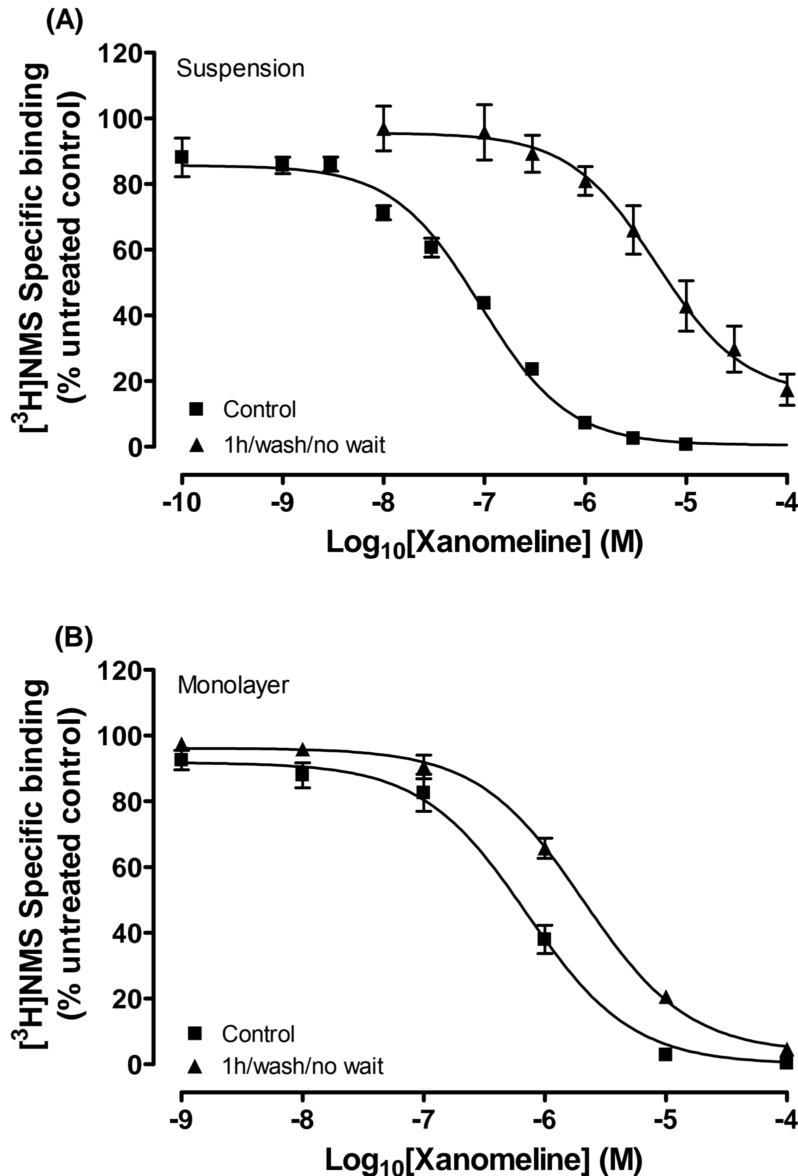


Figure 2. Comparison between inhibition of [^{3}H]NMS binding following xanomeline pretreatments for assays conducted in suspension versus in monolayer in CHO cells stably expressing hM1 receptors. Cells were pretreated with increasing concentrations of xanomeline for 1 hour followed by washing and used immediately (closed triangles). Subsequently, cells were incubated with 0.2 nM [^{3}H]NMS concurrently with xanomeline (closed squares) or following xanomeline pretreatments for 1 hour at 37°C. The radioligand binding assay was conducted in A) suspension or B) monolayer. For (B) individual experiments were normalized to control binding in the absence of xanomeline treatment which had a mean of 13200 dpm/well. Values represent the means ± S.E. of 3-6 experiments conducted in triplicate. Data in (A) provided by Marianne Grant.

Table 1. Effects on $[^3H]$NMS saturation binding parameters when CHO hM3 cells were assayed in monolayer versus suspension. Cells were treated with 10 μM xanomeline for 1 hour followed by washing and immediate use in the binding assay or subsequent to incubation for 23 hours in the absence of free agonist. An additional group of cells was treated with agonist for 24 hours prior to washing. Control untreated or agonist-pretreated cells were then incubated either in monolayer or in suspension with increasing concentrations of $[^3H]$NMS for 1 hour at 37ºC. Parameters (± S.E.M.) were derived from computer-assisted non-linear regression analysis.

	Monolayer[†]		Suspension	
Xanomeline pretreatment group	**K_d [a] (nM)**	**B_{max} [b]**	**K_d (nM)**	**B_{max}**
Control (untreated)	0.42 ± 0.06	67700 ± 3200	0.23 ± 0.01	6100 ± 720
1 h/washout/no wait	3.52 ± 0.52*	78500 ± 1600	0.45 ± 0.01	7000 ± 530
1 h/washout/23 h wait	0.59 ± 0.21	30400 ± 2000*	0.26 ± 0.01	4700 ± 970
24 h/washout/no wait	4.19 ± 0.93*	37000 ± 4600*	1.10 ± 0.32*	5400 ± 610

[a] Equilibrium dissociation constant of $[^3H]$NMS binding.
[b] Cell-surface receptor density (dpm/well).
[†] Values are from saturation binding experiments presented in chapter 3.
*ANOVA followed by Dunnett's post-test comparison detected a significant difference ($p < 0.05$) in K_d or B_{max} between the xanomeline pretreated groups compared with their untreated control.