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MECHANISTIC STUDIES OF THE ALLOSTERIC MODULATION OF MUSCARINIC RECEPTORS

A Dissertation in

Pharmacology

by

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Abstract

This dissertation is the result of a detailed investigation into the effects of the antiarrhythmic drug amiodarone on muscarinic receptors. We have found that amiodarone interacts with muscarinic receptors in a specifically allosteric manner. In radioligand binding studies, amiodarone was only able to partially inhibit the binding of the orthosteric antagonist [^{3}H]N-methylscopolamine (NMS). Additionally, amiodarone was able to alter the rate of dissociation of [^{3}H]NMS from M_1, M_2, and M_5 receptors. These findings suggest that NMS and amiodarone are able to bind to the receptor simultaneously. Furthermore, the pharmacology of the effect on NMS dissociation demonstrated that amiodarone does not interact with the "common" site at which gallamine, obidoxime, and many other muscarinic allosteric ligands are known to bind.

In functional studies, amiodarone enhanced the response stimulated by an acetylcholine concentration that produced about 20% of maximal arachidonic acid release from the M_5 receptor. However, under the same conditions, amiodarone did not enhance M_1 arachidonic acid release. More detailed studies at M_5 found that the effect of amiodarone was to enhance the efficacy of acetylcholine, without increasing its potency. This was the first demonstration of allosteric enhancement of efficacy at the M_5 receptor, and the first demonstration of enhancement of efficacy but not potency at any muscarinic receptor.

Amiodarone also enhanced the maximal level of agonist-stimulated release of arachidonic acid (AA) by the M_3 muscarinic receptor; this enhancement was observed for acetylcholine and for the partial agonist pilocarpine. A similar effect of amiodarone was observed when pilocarpine was used to stimulate inositol phosphate (IP) metabolism, but not when acetylcholine was used. Subsequent studies showed that the IP response exhibited a much larger receptor reserve than the AA response, and reduction of that reserve by receptor alkylation unmasked amiodarone's enhancement of the maximal IP response to acetylcholine. Modulating the receptor reserve also revealed acetylcholine's greater affinity (K_A) for the conformation of the receptor that mediates the AA response.

The amiodarone analog N-ethylamiodarone (NEA) did not alter the maximal agonist response stimulated by M_3, but merely reduced agonist potency (that is, it appeared to be an antagonist). However, the action of NEA could be clearly distinguished from the action of the orthosteric antagonist NMS. Demonstration of this point was facilitated by an elaboration of Hall's allosteric two-state model; this new model represents a system composed of two ligands that compete with each other at the orthosteric site and two ligands that compete with each other at the allosteric site. In conclusion, amiodarone competes with NEA at a novel, extracellular, allosteric site to enhance the maximal stimulation evoked by acetylcholine and pilocarpine in two different responses.

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List of Abbreviations

ACh	acetylcholine
AChEIs	acetylcholinesterase inhibitors
AA	arachidonic acid
ATSM	allosteric two-state model
4L-ATSM	four-ligand allosteric two-state model
CHO	chinese hamster ovary
CTCM	cubic ternary complex model
DMSO	dimethyl sulfoxide
EM-BSA	Eagle's basal medium + 2 mg/ml fatty acid-free bovine serum albumin
GPCR	G protein-coupled receptor
IP	inositol phosphate
NEA	N-ethylamiodarone
NMS	N-methylscopolamine
PAM	positive allosteric modulator
PB	phosphate buffer
PBS	phosphate-buffered saline
PKC	protein kinase C
POB	phenoxybenzamine
TCM	ternary complex model
TSM	two-state model
VU100010	3-amino-N-(4-chlorobenzyl)-4, 6-dimethylthieno[2,3-b]pyridine-2-carboxamide
WIN 62,577	17-hydroxy-17-ethynyl-delta(4)-androstano(3,2-b)pyrimido(1,2-a)benzimidazole
NAM	negative allosteric modulator
W-84	hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide

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writings of Fred Ehlert, Terry Kenakin, Paul Krugman, John Kenneth Galbraith, Robert Heinlein, Issac Asimov, and countless other brilliant authors. I am truly grateful to John for guiding me through my graduate career.

Forward

This dissertation was constructed in an attempt to consolidate the work of two articles, Stahl and Ellis (2010) and Stahl et al. (2011). These papers have been parsed and edited to produce Chapters 2 – 4. It is my hope that this organization provides the reader with a useful explanation of the work we have accomplished.

Chapter 1

Literature Review

1.1 Muscarinic receptors

1.1.1 Cholinergic signaling

Acetylcholine (ACh) is a neurotransmitter in the mammalian central nervous system as well as the periphery (Taylor and Brown, 1999). In autonomic ganglia, the control centers of the autonomic nervous system, ACh is the predominant neurotransmitter. The autonomic nervous system is the nervous system that manages involuntary functions in the body. It is composed of two subdivisions: the sympathetic and parasympathetic nervous systems. These two systems stimulate opposite effects in their target tissues. In the organs innervated by the autonomic nervous system, ACh is the neurotransmitter that conveys parasympathetic tone. In addition to its roles in the central and peripheral nervous systems, ACh stimulates skeletal muscle contraction.

ACh is synthesized in the presynaptic terminal by the enzyme choline acetyltransferase (Taylor and Brown, 1999). Each molecule of ACh is then imported into synaptic vesicles by the vesicular ACh transporter. The packaged ACh is released into the synaptic cleft where it associates with two types of ACh receptors, the nicotinic receptors and the muscarinic receptors. The nicotinic receptors are a family of ligand gated cation channels (Taylor and Brown, 1999). Upon ACh association, these channels facilitate the flow of positive ions into the cell. This ion flux results in changes in the cell's electrochemical gradient. The second type of cholinergic receptors is the family of muscarinic receptors.

1.1.2 Muscarinic receptor properties

The muscarinic receptor family is part of the super family of G protein-coupled receptors (GPCRs). Five subtypes have been identified, designated M_1-M_5 (Kubo et al.,

1986a; Kubo et al., 1986b; Bonner et al., 1987; Bonner et al., 1988). Table 1.1 provides some important characteristics of each subtype (including the pathways to which they couple, the tissues in which they are expressed, and diseases that each subtype may play a role in treating).

Table 1.1 **Muscarinic Receptor Subtypes**

	M_1	M_2	M_3	M_4	M_5
Gα subunit	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$	$G_{i/o}$	$G_{q/11}$
Signal Transduction	Activation of PLC: IP_3/DAG; Ca^{2+}/PKC	Inhibition of adenylate cyclase	Activation of PLC: IP_3/DAG; Ca^{2+}/PKC	Inhibition of adenylate cyclase	Activation of PLC: IP_3/DAG; Ca^{2+}/PKC
Expression Profile	Cortex, hippocampus, striatum, thalamus, sympathetic ganglia, glandular tissue	Hindbrain, thalamus, cortex, hippocampus, striatum, smooth muscle, heart	Cortex, hippocampus, smooth muscle, glandular tissue	Striatum, cortex, hippocampus	Ventral tegmental area
Disease Relevance	Alzheimer's disease, schizophrenia	Alzheimer's disease, Pain	Chronic obstructive pulmonary disorder, incontinence, irritable bowel syndrome	Parkinson's disease, schizophrenia, pain	Drug dependence

Adapted from Langmead et al. (2008b)

The topological organization of the muscarinic receptors resembles that of other GPCRs (Hulme et al., 1990). The defining characteristic of GPCRs is the presence of seven membrane-spanning helices (TM1-TM7). These helices are organized in a counterclockwise configuration when envisioned from the extracellular environment. The N-terminal of the receptor is exposed to the extracellular environment while the C-terminal tail is contained within the cell. The transmembrane domains are linked by amino acid sequences that compose the three extracellular and three intracellular loops. Specific amino acids of the receptor are important for correct folding and function. Two cysteine residues, one in transmembrane domain three and the other in outer loop two, form a disulfide bond (Hulme et al., 1990; Kurtenbach et al, 1990). The importance of these two cysteines is reflected by their high degree of conservation across the entire family of GPCRs. The muscarinic receptors also have glycosylation sites on the N-terminal region and palmitoylation sites on the C-terminal region. These post

translational modifications influence surface expression as well as proper receptor-G protein coupling (van Koppen and Nathanson, 1990). Mutation of the palmitoylation and glycosylation sites in the muscarinic receptors, as well as the adrenergic and bradykinin receptors, causes changes in both agonist potency and efficacy (O'Dowd et al., 1989, Pizard et al., 2001).

Muscarinic receptors are also subject to phosphorylation; receptor phosphorylation is a common mechanism for controlling GPCR activity (Hulme et al., 1990). Receptor phosphorylation is accomplished by kinases which attach phosphate groups to specific serine and threonine residues of the third intracellular loop as well as the C-terminal region. Receptor phosphorylation can have different effects on receptor activity. Agonist stimulation causes phosphorylation at sites that recruit accessory proteins, β-arrestins, and down regulates continued receptor signaling. Phosphorylation at other sites can potentiate certain responses (van Koppen and Nathanson, 1990).

The muscarinic family shares a striking degree of sequence homology. This can be seen from the sequence alignment in Figure 1.1. The M_2 and M_4 receptors are more closely related to each other, by sequence alignment, than to the M_1, M_3, and M_5 receptors; across the family, there is 40% sequence identity (Liao et al, 1989). The N-terminal, C-terminal, and third intracellular loops of the five subtypes are quite different in both length and composition. These regions aside, the sequence identity the family is between 68% - 85% (Bonner et al., 1988). The transmembrane regions of the muscarinic receptors have sequence homology greater than 90%. The dopamine receptor family (another monoamine GPCR) exhibits between 53% - 80% sequence

homology in their transmembrane domains (Missale et al., 1998). The comparison of muscarinic receptors to dopamine receptors highlights the fact that the muscarinic receptors share an unusually high degree of sequence homology in their membrane spanning domains.

The proposed ACh binding pocket is formed in the outer portion of the transmembrane domains. An aspartic acid in TM3 of the M_1 receptor was found to be essential to ACh binding (Fraser et al. 1989). Complementary to the TM3 aspartic acid, a tyrosine in TM7 is also highly conserved in all five muscarinic receptors (Ward et al., 1999). In fact, these two regions are conserved in all monoamine receptors (Hulme et al., 1990). Mutational studies have demonstrated that changes in these regions significantly lower agonist affinity (Fraser et al. 1989, Ward et al., 1999).

1.1.3 Muscarinic receptor activation of G proteins

As stated above, muscarinic receptors belong to the family of GPCRs. These receptors transduce extracellular signals to intracellular signaling pathways by activating GTP-binding proteins (G proteins; Birnbaumer, 1990). The G protein activation cycle is presented in Figure 1.2.

There are twenty-seven different genes that code for different G_α subunits; these G_α subunits can be divided into several groups (Albert and Robillard, 2002). The muscarinic receptors mainly activate G proteins that have one of two different types of G_α. The M_2 and M_4 subtypes activate G proteins that contain the G_α subunit designated G_i. The M_1, M_3, and M_5 subtypes activate G proteins that contain the G_α subunit designated G_q. Additionally, the $G_{\beta\gamma}$ component of the activated G protein has been shown to modulate cellular targets (Caulfield, 1993).

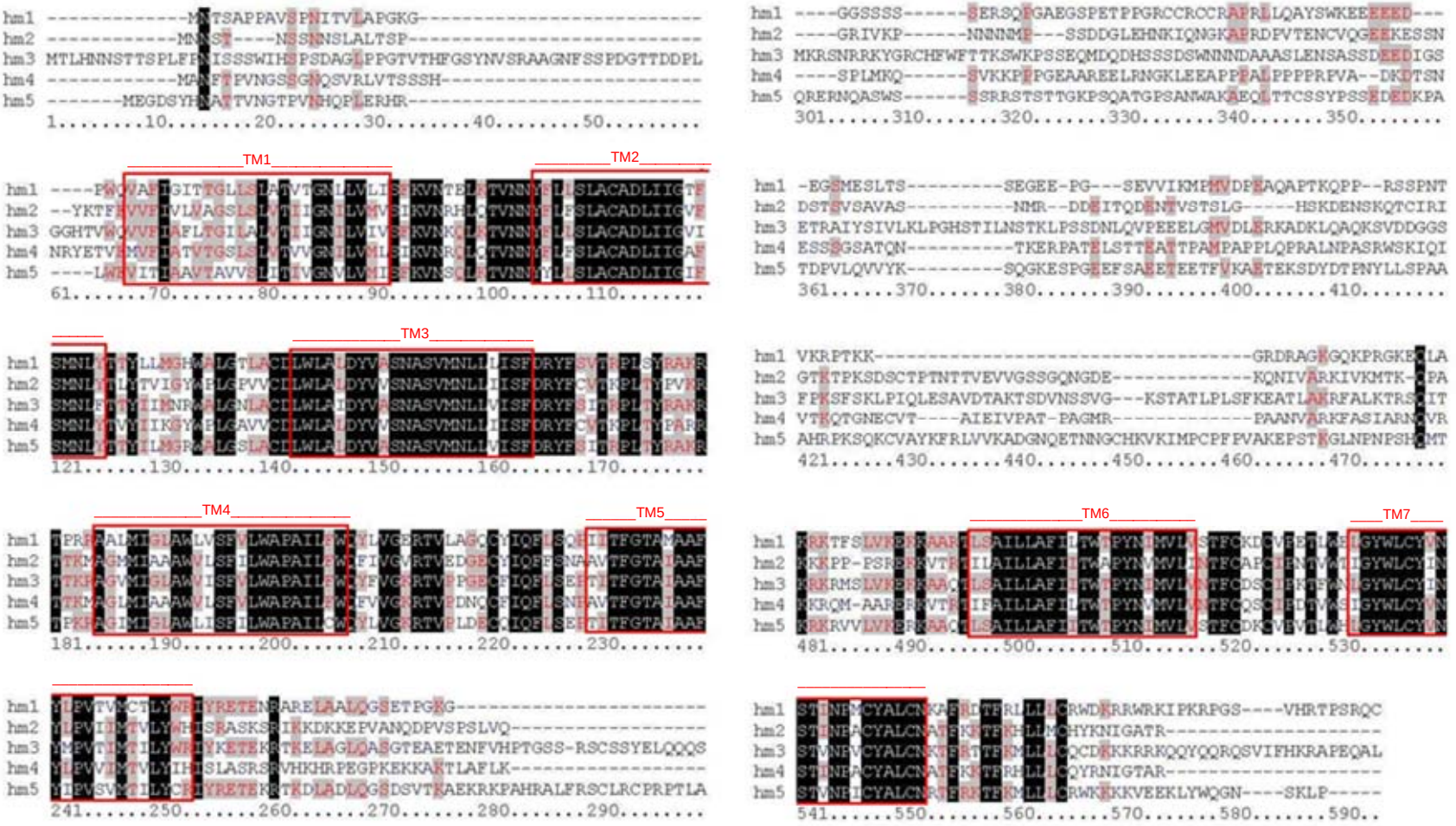
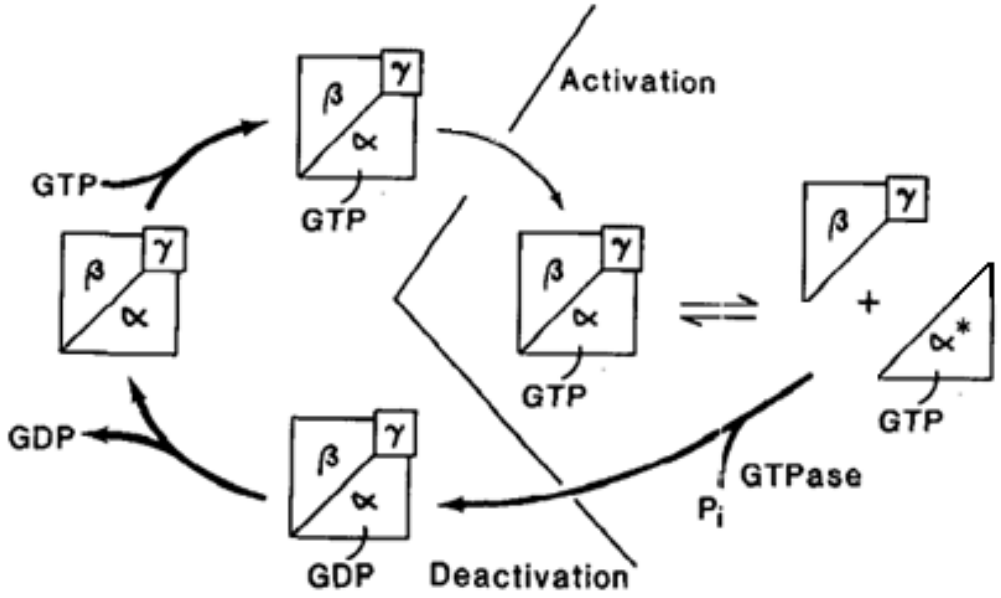


Figure 1.1. Amino acid sequence alignment of the five human muscarinic receptors. The red boxes indicate the transmembrane domains of the receptor. The amino acids in black boxes designate residues that are conserved across the muscarinic receptor family; the amino acids in red are conserved between some members of the family.



Adapted from Birnbaumer, 1990

Figure 1.2. The G protein activation cycle. G proteins are composed of one G_α, one G_β, and one G_γ subunit which associate to form a heterotrimeric complex. In the ground state, GDP is bound to the G_α subunit and the G_α and $G_{\beta\gamma}$ subunits are associated. When the receptor adopts an active conformation it causes GDP to dissociate and GTP to associate with the G_α subunit. The GTP-bound G_α subunit then separates from the $G_{\beta\gamma}$ subunit and both subunits have effects on intracellular targets. The GTP-bound G_α becomes inactive when GTP is hydrolyzed to GDP and the inactive GDP-bound G_α reassociates with $G_{\beta\gamma}$ to complete the G protein cycle.

1.1.3.1 M_2 and M_4 signaling

The M_2 and M_4 receptors inhibit the activity of adenylyl cyclase through activation of G_i. Adenylyl cyclase is responsible for the generation of cyclic adenosine monophosphate (cAMP), an important signaling molecule. cAMP is the product of adenylyl cyclase metabolism of adenosine triphosphate and modulates intracellular kinases (specifically cAMP dependent protein kinase) and ion channels (Alberts, 2002).

1.1.3.2 M_1, M_3, and M_5 signaling

M_1, M_3, and M_5 receptors activate the G_q subunit which stimulates phospholipase C (Exton, 1996). Phospholipase C catalyzes the metabolism of a specific membrane lipid, phosphatidyl-inositol 4,5-bisphosphate. Through cleavage of the phosphate bond that attaches the inositol headgroup to the lipid, the enzyme produces two products: inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). These two second messengers then act on their respective downstream cascades. IP3 acts on receptors on the endoplasmic reticulum to stimulate the release of intracellular calcium. DAG causes the activation of protein kinase C (PKC).

Both the elevation of intracellular Ca^{2+} and PKC activation cause instantaneous and prolonged changes in the cellular environment (Lanzafame et al., 2003). The instantaneous effects include depolarization of the cell membrane caused by increased intracellular calcium. The prolonged changes include the activation of transcription factors such as mitogen-activated protein kinase (Nagao et al, 1998).

1.1.4 Knockout Mice

A number of agonists and antagonists have been discovered that are selective for the muscarinic family. Unfortunately, the highly conserved nature of the orthosteric acetylcholine binding pocket has prevented the development of truly subtype selective muscarinic ligands. Some tissues in the periphery express a subset of muscarinic receptors. For instance, the heart only expresses the M_2 receptor. However, all five muscarinic receptors are expressed in the CNS and this has muddled the correlation of individual receptors with their function in the brain (Levey, 1993). Our current understanding of the physiological and behavioral role of the individual subtypes has largely relied on studies in knock-out (KO) mice. With KO mice it has been possible to make some conclusions on the physiological role of individual muscarinic subtypes.

1.1.4.1 M_1 Knockout Mice

The M_1 receptor is expressed in the periphery in salivary glands where it controls secretion (Levey, 1993). M_1 has been associated, as shown by *in vivo* experiments, with pilocarpine-induced seizure. M_1 KO mice are not susceptible to pilocarpine-induced seizure (Hamilton et al., 1997). This finding was specific for M_1 KO mice; M_2-M_5 KO mice are susceptible to pilocarpine-induced seizure (Bymaster et al., 2003). Due to the expression profile of M_1 in the forebrain, M_1 was expected to be involved in memory and cognition. M_1 KO mice performed normally in several measures of working memory and cognition (Miyakawa et al., 2001). In contrast, M_1 KO mice exhibited impairment in other tests of memory and consolidation (Anagnostaras et al., 2003). In other studies of M_1 KO mice it was found that the effect of agonist administration in the hippocampus and cortex on several response cascades was attenuated (Berkeley et al.,

2001; Hamilton and Nathanson, 2001). The studies in KO mice suggest that the M_1 receptor is influential but other subtypes of muscarinic receptors are also involved in memory and cognitive function.

1.1.4.2 M_2 Knockout Mice

In the periphery, the M_2 receptor plays a major role in controlling heart rate. M_2 KO mice do not exhibit bradycardia when challenged with muscarinic agonists (Fisher et al., 2004). The M_2 receptor also modulates smooth muscle contraction in several organs including the stomach, bladder, and trachea (Stengel et al., 2000). In the spinal cord, muscarinic mediated analgesia was greatly attenuated, but not eliminated, in M_2 KO mice (Gomeza et al., 1999). In combined M_2/M_4 KO mice, the analgesic effects of oxotremorine were completely absent; implicating both receptor subtypes in cholinergic mediated analgesia (Duttaroy et al., 2002). In higher brain regions, the M_2 receptor was found to influence memory. M_2 KO mice exhibited significant deficits in several learning tests (Seeger et al., 2004). In a different role, the M_2 receptor acts as an autoreceptor in both central (Zhang et al., 2002a) and peripheral locations (Zhou et al., 2002). Autoreceptors provide feedback on neurotransmitter levels in the synapse to prevent excessive stimulation.

1.1.4.3 M_3 Knockout Mice

The M_3 receptor has a wide expression profile in the periphery. It plays a major role in controlling smooth muscle tone in several locations in the body. M_3 KO mice reflect this in limited contractility in the urinary bladder and numerous other smooth muscle-containing tissues (Matsui et al., 2000). These animals suffered from severely distended bladders and enlarged pupils. Pupil distension was dramatic, but incomplete,

as atropine caused further dilation and light caused some constriction. The M_3 receptor also regulates gastric and salivary secretions. Although expressed centrally, the role of the M_3 receptor in the CNS has remained enigmatic.

1.1.4.4 M_4 Knockout Mice

The M_4 receptor is expressed in the CNS, predominantly in the forebrain (Levey, 1993). This limited expression may make it a useful drug target. From studies of KO mice, it has been implicated in the regulation of striatial dopamine release (Zhang et al., 2002b). This suggests that the M_4 receptor plays a role in modulating locomotor activity and may make it an attractive target for the treatment of Parkinson's disease. This disease is linked to dysregulation of striatial dopamine signaling. There is also evidence that M_4, along with M_2, plays a role in analgesia. Although M_2 is believed to be the prominent receptor involved in producing analgesia, it has been shown that M_4 contribution is also significant and M_4 is still considered an attractive target for treating pain (described above). This point is further substantiated by to the limited expression profile of M_4 which would make it an attractive target (potentially it may be devoid of peripheral side effects).

1.1.4.5 M_5 Knockout Mice

The M_5 receptor, like the M_4 receptor, has a limited expression profile that appears to be largely confined to the CNS. For this reason, the M_5 receptor may be a potential therapeutic target. M_5 deficient mice are resistant to drug dependency as demonstrated by the reduction of withdrawal symptoms following opioid administration (Basile et al., 2002). This makes the M_5 receptor an attractive target for the treatment of addiction. The M_5 receptor also induces vasodilation in arteries that feed the CNS

(Yamada et al., 2001). M_5 deficient mice demonstrated a loss of cholinergic-mediated vasodilation in cerebral arteries.

1.1.5 Pathologies associated with muscarinic receptors

1.1.5.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease which currently affects more than 24 million people worldwide with the expectation of 4.6 million new cases, annually (World Health Organization, 2006). The loss of cholinergic signaling in the hippocampus and cortex is associated with the progressive loss of memory and the development of cognitive impairment (Muir, 1997). In line with this reasoning, most of the current pharmacologic treatments for AD are acetylcholinesterase inhibitors (AChEIs). AChEIs cause elevation of ACh levels by inhibiting the enzyme responsible for its degradation, acetylcholinesterase. Although effective, AChEIs have undesirable side effects and are only effective for treating the early stages of disease progression.

Two of the hallmark characteristics of the disease are the development of amyloid plaques and neurofibrillary tangles. Amyloid plaques are composed of an improperly processed component of the amyloid precursor protein, the Aβ peptide. The improperly processed peptide then aggregates to form amyloid plaques. Aβ has been shown to have cytotoxic effects on cholinergic neurons and it has been observed that plaque formation and disease progression appear to occur in parallel (Fisher et al., 2003); amyloid plaque formation may be a causative factor in the development of neurofibrillary tangles (Langmead et al., 2008b).

The M_1 receptor is a potential target for the treatment of AD. Treatment with AChEIs causes elevation of ACh levels and M_1 may be the direct beneficiary of this

increase in ACh levels. Selective M_1 agonists may provide similar benefits without the undesirable side effects of AChEIs. Some evidence suggests that M_1 agonists may directly influence progression of the disease. One of the effects of M_1 stimulation is the elevation of PKC activity. In several studies, the development of Aβ plaques was inhibited by PKC. Targeting M_1 receptors, through their ability to induce PKC activity, may be useful in preventing the symptoms as well as treating the underlying cause of AD.

M_2 receptor antagonists may also be beneficial in treating AD. The M_2 receptor is present on the presynaptic surface where it acts as a feedback inhibitor on further ACh release. Inhibition of this receptor may elevate ACh levels in the synapse in a manner similar to AChEIs. In this case, M_2 antagonists may be more effective because they would act specifically at M_2-containing synapses. This approach would selectively predispose the M_2-containing synapses to developing higher concentrations of ACh instead of the global elevation of ACh levels caused by AChEIs. M_2 antagonists would also allow endogenous spatial-temporal patterning to remain largely intact because synapse firing would still control ACh levels.

1.1.5.2 Schizophrenia

Schizophrenia is a chronic psychiatric disorder characterized by positive (hallucinations) and negative (social withdrawal and cognitive deficits) symptoms. Over activity in the forebrain dopamine systems is believed to be responsible for some of these symptoms. This conclusion is based on the effectiveness of dopamine receptor antagonists in the clinic. Unfortunately, these antagonists are only modestly effective and cause undesirable side effects.

Cholinergic pathways have been implicated in schizophrenia. In the forebrain of individuals with schizophrenia, the expression of muscarinic receptor is diminished. Furthermore, a polymorphism in the M_1 receptor has been shown to correlate with individuals exhibiting prefrontal cortical dysfunction (Langmead et al., 2008b). Other studies have demonstrated muscarinic receptor expression deficits in the forebrain. In line with these findings, AChEIs have been shown to relieve some symptoms of schizophrenia.

M_1 and M_4 agonists may have utility in some symptoms of schizophrenia. M_1 receptors play a role in cognitive processes as well as being implicated in modulating dopamine systems. M_4 receptors in the midbrain are believed to act as autoreceptors at synapses which feed dopamine pathways in the forebrain. The increase in forebrain dopamine levels is believed to cause some of the psychosis associated with schizophrenia. Therefore, treatment with M_4 agonists may alleviate some of this over stimulation.

1.1.5.3 Parkinson's Disease

Dopamine tracts of the midbrain play a central role in the coordination of movement and are linked to Parkinson's disease (PD). In PD, the progressive loss of dopamine signaling results in the development of debilitating dyskinesia. Dopamine replacement therapy is currently the most effective approach for PD treatment. ACh is also believed to play a role in PD (Mayorga et al., 1999). The dysfunction in dopamine pathways shifts the balance of signaling to favor cholinergic influence. This is due to the coexpression of dopamine and muscarinic receptors, specifically M_4, on neurons in the nigrostriatal pathway. M_4 antagonists may reset this balance. M_4 KO mice

demonstrated elevated levels of basal locomotor activity as well as elevated levels of locomotor activity stimulated by dopamine receptor agonists. This suggests that M_4 antagonism may facilitate more effective dopamine signaling.

1.2 Receptor theory

1.2.1 Receptors

The basis of receptor pharmacology was developed by J.N. Langley and A.J. Hill. In a paper on the studies of nicotine-stimulated muscle contraction Langley proposed the concept of receptors (Langley, 1905). He suggested from these studies that two cellular components could be distinguished. One component, the *chief substance*, was responsible for performing the chief function of the cell. The second component, the *receptive substance*, conveyed the chemical signal (nicotine) to activate the *chief substance*. Langley postulated that the action of the chemical signal was through its combination with the "receptive substance". Hill (1909) further developed Langley's idea by demonstrating that the stimulation of muscle contraction by nicotine could be described using the mass-action equation. The underlying principle behind this equation is that specific binding sites exist for nicotine in the tissue.

The next major development in receptor pharmacology was the recognition of competitive antagonists. Competitive antagonists are ligands which do not activate the receptor but compete for the orthosteric site. The orthosteric site is the binding site for the endogenous ligand. Clark and Raventos (1937) and Gaddum (1937) both recognized the mechanism of competitive antagonists but the work of Schild provided the most useful insight on competitive antagonists. Schild (1949) demonstrated that antagonists affect response curves in a defined manner. It was recognized at that time that competitive antagonists cause the agonist response curve to shift rightward. Schild's work concluded that the degree of this curve shift was related to the ratio of the antagonist concentration to the antagonist's affinity ($[B]/K_B$). He demonstrated this by

graphing data in what is now known as the Schild plot. In the Schild plot, log[dose ratio-1] is presented on the *y*-axis and the log[antagonist] is presented on the *x*-axis. The dose ratio can most easily be represented as the ratio of the agonist EC_{50} in the presence of a competitive antagonist to the control agonist EC_{50} (EC_{50} with antagonist / control EC_{50}). By representing the response curves in this way, Schild demonstrated that there is a linear relationship between the change in the log of the dose ratio and the log of the antagonist concentration if the antagonist binds to the orthosteric site.

1.2.2 The response function

The initial studies of drug action were founded on the premise that response was directly proportional to receptor occupancy. Simply stated, each agonist binding event contributed equally to the total response. It was the work of Robert Stephenson that marked a major paradigm shift in pharmacology. Stephenson's insight developed from a structure-activity relationship he published on different analogs of the agonist trimethylammonium (Stephenson, 1956). In this study, he demonstrated that these analogs stimulated different levels of maximum response. This led Stephenson to propose the existence of a parameter which describes the signaling ability of the ligand-associated receptor. He called this parameter efficacy and defined it as the ability of different drugs to have "varying capacities to initiate a response". Under this framework, it was only a small step to recognize that different agonists may stimulate different levels of response at equivalent occupancy. Explicitly segregating occupancy from response was the catalyst for two major theory advances: receptor reserve and the two-state model.

1.2.3 Receptor reserve

> "*The evidence that receptor occupancy is not necessarily the limiting factor in determining the magnitude of a tissue response suggests that all theoretical deductions from mass-action analysis of dose-response curves should be accepted with caution.*"
>
> -Mark Nickerson (1956)

Receptor reserve defines the condition where an agonist can stimulate maximal response although it does not occupy all of the available receptors. For example, a concentration of an agonist may occupy a small number of receptors yet stimulate maximal response. In contrast, a different agonist may occupy all available receptors and not attain maximal response. Although the efficacy of the two agonists is different (they stimulate different degrees of maximal response), the size of the receptor population plays a role in the maximal response. If the partial agonist described above was presented to a different tissue, with a larger receptor population, it may achieve maximal response.

Furchgott (1955) presented a method of analysis for receptor reserve. This analysis was based on the finding that the receptor reserve could be altered by treatment with an irreversible antagonist. Nickerson and Goodman (1947) provided the instrument for receptor reserve elimination with the discovery of the first irreversible antagonist (dibenamine). The presence of a receptor reserve causes an agonist's concentration-response curve to lie to the left of its occupancy curve. (The agonist's potency is greater than its affinity.) Irreversible antagonists inactivate receptors by covalently binding to the receptor's orthosteric site. These inactivated receptors have, therefore, been removed from the available pool of receptors that can be stimulated by an agonist. Treatment with an irreversible antagonist would cause the agonist's response curve to shift rightward (and approach its occupancy curve).

Irreversible antagonists cause two different effects on response curves. One effect of the irreversible antagonist is to cause the agonist's response curve to be shifted rightward. With the loss of spare receptors, higher concentrations of agonist are necessary to occupy the same number of receptors occupied in the control curve. The other effect is to cause a decrease in maximal response. The irreversible antagonist removes receptors and, at some point, the remaining receptors cannot achieve maximal response. In order to understand this, it should be recognized that a certain number of receptors are necessary to stimulate maximal response. Furchgott concluded that, as maximal response is lost, the EC_{50} of the response curve approaches the actual affinity of the agonist for the receptor.

1.2.4 The two-state model

An important advancement in receptor pharmacology was the development of the two-state model (Figure 1.3). In a 1951 paper, Jeffries Wyman stated that the oxygenation of hemoglobin causes a "change of conformation of the hemoglobin molecule" (Wyman and Allen, 1951). This concept was further developed by the publication of the two-state model of receptor activation (Del Castillo and Katz, 1957; reviewed in Leff, 1995).

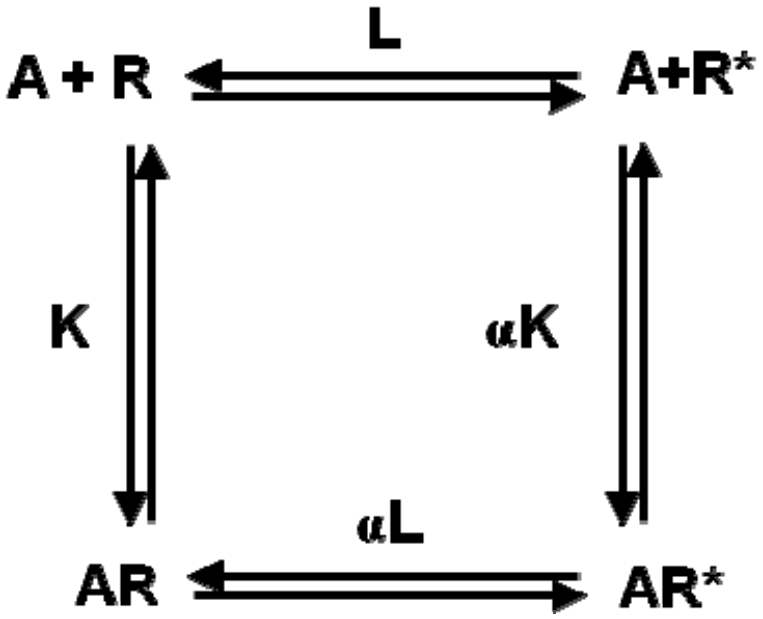


Figure 1.3. The two-state model (Adapted from Leff, 1995)

According to the model, the receptor can exist in two activity states: the inactive state and the active state. The model utilizes these two states to explain how efficacy can be ligand specific. In the model, a ligand has two separate affinity values; one for each receptor state. The efficacy of a ligand can be defined as the relative affinity of the ligand for the two states. An agonist with only a slightly greater affinity for the active state will have less efficacy, whereas an agonist with a much greater affinity for the active state will exhibit greater efficacy. A ligand which has the same affinity for the active and inactive state will act as a competitive antagonist.

In order to provide a complete picture of how the two-state model handles efficacy it is necessary to discuss inverse agonists. In the two-state model, the receptor can exist in four configurations: the inactive receptor (R), the active receptor (R*), the ligand-bound inactive receptor (AR), and the ligand-bound active receptor (AR*). The distinction of R* provides a condition where the receptor is active but not associated with the ligand. This circumstance is defined as constitutive activity. It was originally

discovered by Costa and Herz (1989); they also demonstrated that ligands can possess negative efficacy. That is, constitutive activity can be inhibited by the association of certain ligands (which they named inverse agonists). The two-state model elegantly assimilates this finding by predicting inverse agonists will have greater affinity for the inactive state of the receptor than the active state of the receptor.

1.2.5 The ternary complex model

GPCR signaling acts through the activation of heterotrimeric G proteins. The ternary complex model is a simple mechanism for describing this form of coupling (De Lean et al, 1980; Figure 1.4). It assumes that the receptor acts as a focal point; the receptor's extracellular face binds the ligand and the receptor's intracellular face binds the heterotrimeric G protein. The ternary complex model recognizes the existence of two distinct binding events. This model addresses the fact that that these binding events do not happen in isolation, but rather one binding event may modulate the other binding event. The relationship between the two binding events is expressed in the model by the parameter γ (binding cooperativity) which can best be explained with the following example.

When the receptor is associated with the G protein the affinity of the ligand may be different from the affinity of the ligand for the G protein-deficient receptor. This is a reciprocal relationship; if G protein association modulates ligand binding then ligand binding modulates G protein association to the same degree. Indeed, GPCR binding studies have demonstrated just such a relationship. Agonist binding curves typically exhibit shallow slopes and this shallowness is attributed to the presence of two sites: low affinity and high affinity binding sites. Inclusion of GTP in binding studies causes

these binding curves to assume the form representative of a single form of the receptor (the form of the receptor without the G protein associated). It is assumed that the effect of GTP is to cause the dissociation of the G protein from the receptor and therefore agree with a single site model (Christopoulos and El-Fakahany, 1999).

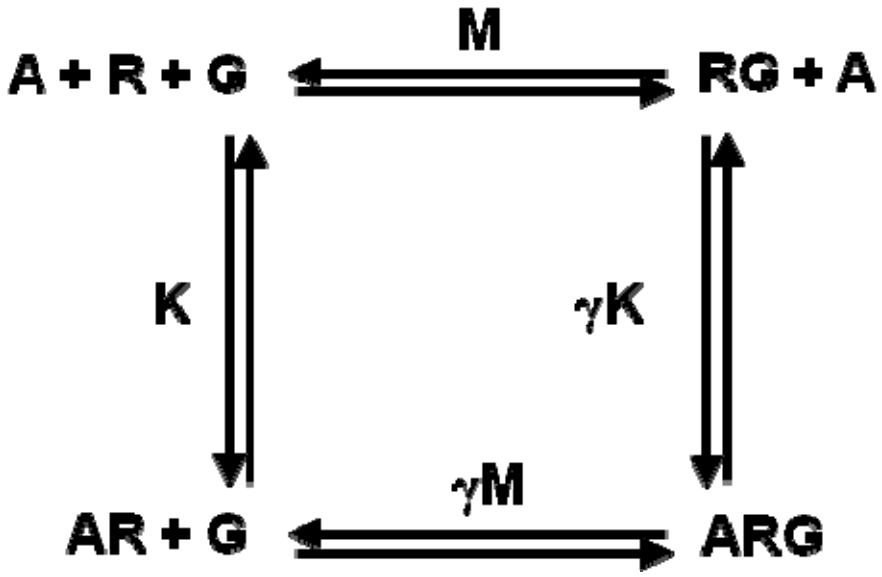


Figure 1.4. The ternary complex model (Adapted from Christopoulos and Kenakin, 2002)

1.3 Allosteric modulation of receptors

1.3.1 The allosteric ternary complex model

Stockton et al. (1983) applied the ternary complex model (Figure 1.4) to describe the effects of gallamine on M_2 receptors in the heart. They proposed the existence of an allosteric site on the receptor which was distinct from the orthosteric acetylcholine binding site. In support of this hypothesis, they performed Schild analysis of gallamine inhibition of [^{3}H]NMS binding. The effects of gallamine were shown to be saturable (non-linear) with the dose-ratio of gallamine reaching an upper limit. This finding is inconsistent with gallamine occupying the orthosteric acetylcholine binding site but is entirely consistent with gallamine occupying a distinct allosteric site. If the ternary complex model (presented in Figure 1.4) is reinterpreted under the assumption that G (originally the G protein) is instead representative of a second ligand, the ternary complex described is then composed of the receptor and two ligands.

1.3.2 The allosteric two-state model

In 2000, David Hall published the allosteric two-state model (Hall, 2000; Figure 1.5). It is a mechanistic model of allosteric modulation of response. (The construction of this model, and the manner by which it incorporates the two-state model and the ternary complex model, is presented graphically in Figures 1.S1-S3 at the end of this section) In the model, A is the ligand that associates with the orthosteric site and B is the ligand that associates with the allosteric site. The model defines response as the fraction of receptors which are stabilized in the active state (analogous to the two-state model). Four active receptor states are predicted: R* (constitutively active), AR* (orthosteric ligand bound), R*B (allosteric ligand bound), and AR*B (orthosteric ligand

and allosteric ligand bound). The same four binding conditions exist with the receptor in the inactive state (R, AR, RB, and ARB). The transitions among the bound/unbound and active/inactive receptor states are influenced by three equilibrium constants (these constants are defined as K, M, and L). These three constants define the affinity of each ligand for the receptor (K and M) and the intrinsic activation constant of the receptor (L). Four cooperativity parameters are also defined (α, β, γ, and δ) which describe how the binding of ligands may modify the three parameters.

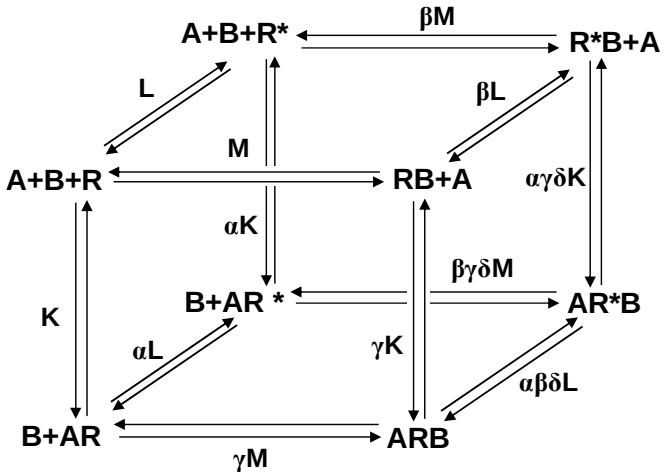


Figure 1.5. The allosteric two-state model (Adapted from Hall, 2000)

Two of these cooperativity parameters are fairly intuitive. The *intrinsic efficacy* of the orthosteric and allosteric ligands are defined by the parameters α and β. The intrinsic efficacy is the ability of the ligand to select for the active species of the receptor over the inactive receptor species. (This is exactly the same as the affinity ratio that the **two-state model** uses to define efficacy; see *Section 1.2.4*) The binding cooperativity that may exist between the orthosteric ligand and the allosteric ligand is defined by the parameter γ. (Again, this is exactly the same as the γ defined in the **allosteric ternary complex model**; see *Section 1.3.1*). The δ parameter is more complicated. δ is defined as the activation cooperativity that exists between the two ligands. This form of

cooperativity provides for the circumstance where the ternary complex (composed of orthosteric ligand- receptor-allosteric ligand; AR*B) exhibits a different efficacy from the other active states of the receptor (AR* and R*B). For example, it is possible to postulate the existence of two theoretical ligands (one orthosteric and one allosteric) where each ligand alone has neutral intrinsic efficacy ($\alpha = \beta = 1$). In this case, the binding of either of these ligands will not produce a response. However, if the activation cooperativity, δ, that exists between these ligands was greater than one, it would indicate that the ternary complex formed by the receptor and both ligands is capable of stimulating a response.

1.3.3 Potential benefits of allosteric modulation

There may be several advantages to employing allosteric modulators instead of conventional orthosteric ligands in medicine (Christopoulos and Kenakin, 2002). Due to their noncompetitive nature, they may be safer to administer. They may not directly cause receptor stimulation or block endogenous signaling. Their noncompetitive nature also predisposes them to a ceiling effect. If greater quantities of an allosteric ligand are administered its effect will reach a limit as the sites it binds to become saturated. In contrast, if greater quantities of an orthosteric ligand are administered then greater effects are achieved.

Another potential benefit of allosteric modulators is that they may provide greater binding site selectivity than orthosteric ligands. This is especially true for the muscarinic receptors. The highly conserved nature of the orthosteric site has impeded the development of truly subtype selective orthosteric ligands. It is believed that allosteric

sites are less well conserved between muscarinic subtypes and therefore may be better therapeutic targets.

A third potential benefit of allosteric modulators is that they may exhibit different binding cooperativities for different receptor subtypes. In this case, the allosteric ligand may bind to all receptor subtypes but different binding cooperativities could mean that the allosteric modulator enhances the potency of an orthosteric ligand at one subtype but has no effects on the orthosteric ligand at the other subtypes. This is the absolute selectivity that was defined by Birdsall et al. (1999).

Finally, allosteric modulators may affect receptor activity without disrupting the pattern of synaptic signaling. This effect is specific for allosteric modulators; ligands that associate with the orthosteric site (agonists and competitive antagonists) diminish the influence of neurotransmitter release by occupying the orthosteric binding site. Allosteric modulators do not occupy the orthosteric binding site (the site that the neurotransmitter binds). Instead, allosteric modulators act cooperatively with the neurotransmitter to influence signaling (Christopoulos and Kenakin, 2002). In this way, the allosteric modulator may influence receptor activity while the pattern of spatial and temporal synaptic signaling is not altered.

1.3.4 Allosteric modulators of muscarinic receptors

Attempts to develop muscarinic orthosteric ligands with significant subtype specificity have not been successful. This is believed to be due to the sequence homology at the orthosteric site. The possibility exists that allosteric sites, sites physically distinct from the orthosteric site, may provide more useful targets for subtype specific ligand development. Interestingly, due to the lack of selective ligands, the

muscarinic receptors have played a significant role in the advancement of allosteric theory.

1.3.4.1 Allosteric modulators of potency

The first muscarinic allosteric ligand to be discovered was gallamine. Stockton et al. (1983) demonstrated that the effects of gallamine were not competitive. The allosteric effects of gallamine are an example of negative cooperativity. In binding studies, gallamine caused incomplete inhibition of antagonist binding. They also demonstrated that there was a limit to the rightward shift of antagonist binding curves caused by gallamine. These two findings allowed the researchers to conclude that gallamine associates with an allosteric site on muscarinic receptors, to inhibit binding, and does not associate with the orthosteric site.

Tucek et al. (1990) reported that alcuronium, another muscarinic allosteric modulator, was positively cooperative with the antagonist NMS. The first report of positive cooperativity with ACh at muscarinic receptors came from Lazareno et al. (1998) and Birdsall et al. (1999). This work demonstrated that brucine and its analogs could cause enhancement of ACh affinity. Their work also demonstrated that positive allosteric modulation could be accomplished in a subtype specific manner. Brucine exhibited positive allosteric cooperativity with ACh specifically at the M_1 receptor although it was able to bind to other subtypes. This led the authors to define a novel form of selectivity, absolute subtype selectivity. Absolute subtype selectivity is present when the allosteric ligand binds to all receptor subtypes but only enhances agonist potency at one subtype. In this case, although the allosteric modulator occupies the allosteric site on the other subtypes, it has neutral allosteric effects on these receptors.

BQCA is a highly selective M_1 positive allosteric modulator (Shirey et al., 2009). In response studies, BQCA caused significant enhancement of ACh potency at the M_1 receptor while not affecting the other muscarinic subtypes. In animal studies, BQCA caused significant elevation of markers of receptor activity (Ma et al., 2009). These findings indicate that BQCA has significant potential for selectively targeting M_1 receptors. Other groups have developed allosteric ligands which are selective for the other muscarinic subtypes (Bridges et al., 2009; Lazareno et al., 2004).

1.3.4.2 Allosteric modulators of efficacy

VU100010 causes ACh potency to be enhanced at the M_4 receptor. Its ability to enhance potency is subtype specific; it does not affect the other muscarinic subtypes (Shirey et al., 2008). VU100010 was also demonstrated to cause elevation of M_4 efficacy in several measures of response. VU0029767, an M_1-selective allosteric modulator, was found to cause potentiation of ACh mediated response (Marlo et al., 2009). The effect of VU0029767 was the result of the elevation of agonist potency and efficacy. The authors suggest that the stimulation of efficacy may be reflective of the ability of the allosteric ligand to selectively stabilize certain active conformations of the receptor. In agreement with this hypothesis, VU0029767 causes potentiation of one measure of receptor-mediated response, IP metabolism, while not affecting another, phosopholipase D activity.

1.3.4.3 Allosteric agonists

The compound AC-42 was demonstrated to selectively stimulate M_1 receptors (Spalding et al., 2002). It did not cause stimulation of any of the other muscarinic receptors. Mutation studies determined that the AC-42 agonist activity is not conveyed

through the orthosteric site. Rather it activates response through an allosteric site on the M_1 receptor. The selective effects of AC-42 provided the authors with an avenue for determining the location of the allosteric site. Through the use of M_1/M_5 chimeras, they were able to show that the allosteric site was defined by regions of extracellular loop three, the N-terminal, and the upper regions of TM1 and TM7. Since this initial publication, other muscarinic allosteric agonists have been discovered (Spalding et al., 2006; Jones et al., 2008; Langmead et al., 2008a).

1.3.4.4 Allosteric binding sites

The muscarinic receptors exhibit multiple allosteric binding sites. The first of these to be classified was the gallamine site. This site is also the binding site for the allosteric modulators alcuronium, TMB-8, obidoxime, and tacrine. For the M_2 receptor, this allosteric site consists of the EDGE region (Glu^{172} - Asp^{173} - Gly^{174} - Glu^{175}) of the third extracellular loop as well as part of TM6 (Leppik et al., 1994; Ellis et al., 1993). Lazareno et al. (2000) demonstrated that staurosporine and the "KT" compounds shared an allosteric site which was distinct from the gallamine site. The discovery of muscarinic allosteric agonists and the demonstration that their effect is through receptors regions that do not overlap with the gallamine site further supports the conclusion that multiple allosteric sites exist (Spalding et al., 2002).

1.4 Rational and Hypothesis

Muscarinic receptors have significant potential for the treatment of Alzheimer's disease, Parkinson's disease, and schizophrenia. The high degree of sequence homology at the muscarinic orthosteric site has hindered the development of highly subtype-selective ligands. A number of ligands have been developed that act as orthosteric agonists and antagonists of muscarinic receptors. Unfortunately, these ligands produce undesirable dose-limiting side effects because of their lack of selectivity. It is reasonable to suggest that allosteric ligands may provide greater subtype specificity and avoid the side effects associated with these nonselective ligands. In recent years a number of muscarinic allosteric ligands have been developed and, in some cases, these ligands are selective for specific subtypes.

Amiodarone is a drug used in the clinic to treat cardiac arrhythmias. Its mechanism of action is believed to be through the inhibition of cation currents in the heart (Kodama et al., 1997). Other groups have found that amiodarone has the ability to modulate other targets including adrenergic and thyroid hormone signaling (Yin et al., 1994). In a preliminary investigation we screened a number of potential allosteric modulators to evaluate their effects on [^{3}H]arachidonic acid release. The results of this drug screen led us to hypothesize that amiodarone modulates response through an allosteric site on muscarinic receptors.

The work presented in this dissertation is focused on understanding the allosteric effects of amiodarone on muscarinic receptors. The ability of amiodarone to cause the enhancement of agonist efficacy is a rather unique form of modulation. Subsequently, we evaluated amiodarone's allosteric effects in the [^{3}H]inositol phosphate (IP)

metabolism response. Amiodarone caused enhancement of the efficacy of pilocarpine but did not affect the efficacy of ACh. The ability of amiodarone to potentiate the efficacy of both agonists in AA release but only one agonist in IP metabolism led us to hypothesize that IP metabolism may be subject to a ceiling on maximal response. This ceiling effect in IP metabolism would be reflected by the presence of a receptor reserve. Our analysis sought to determine the degree of receptor reserve and the influence of the receptor reserve on allosteric modulation of maximal response. Additionally, we investigated the hypothesis that amiodarone and an analog of amiodarone compete for a common allosteric site. In order to pursue this hypothesis we developed a model of allosteric modulation of response. This model was able to describe the effect of competition at the allosteric site in response. In summary, the effects of amiodarone on muscarinic receptor-mediated response were determined to be through an allosteric mechanism. This mechanism involves the allosteric modulation of agonist efficacy.

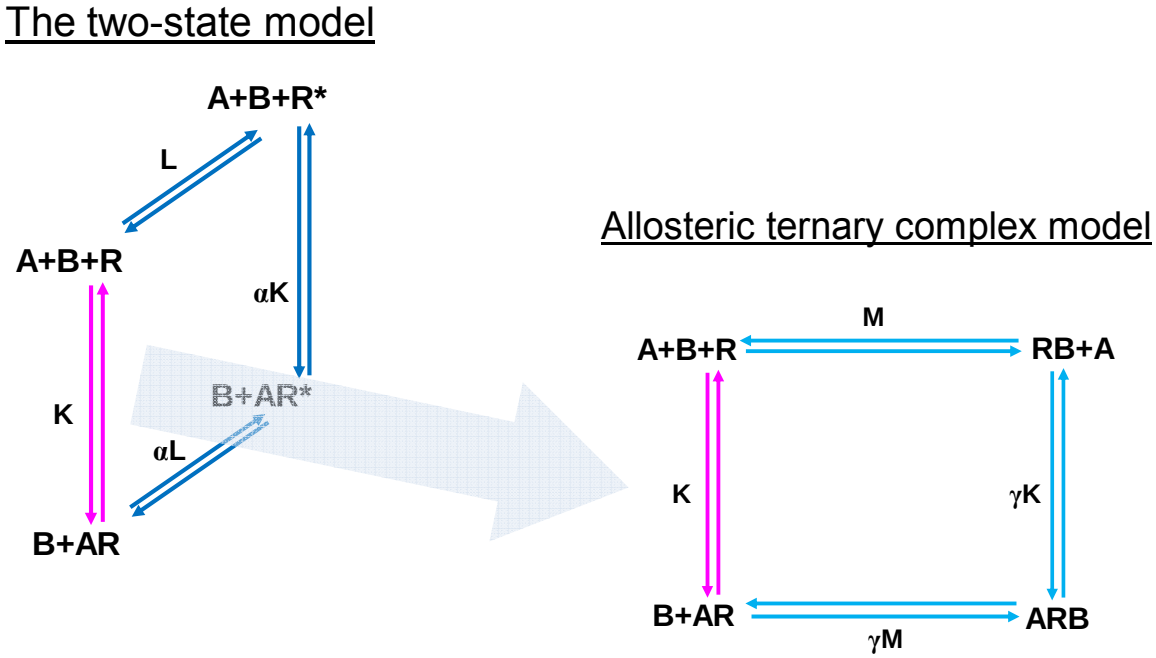


Figure 1.S1. The two-state model and the allosteric ternary complex model. The association of the ligand A is a component of both of these models.

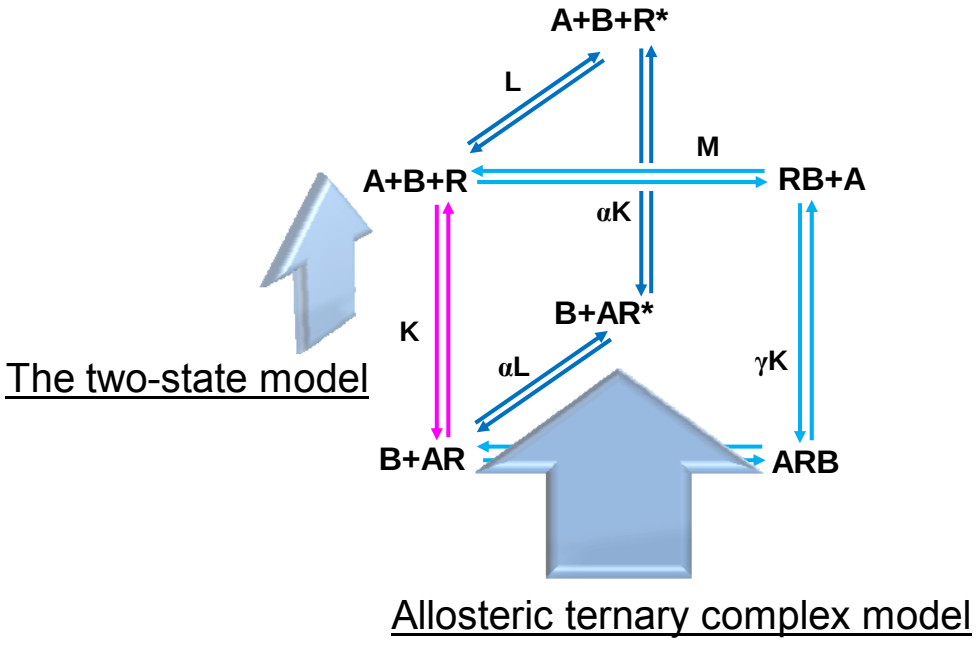


Figure 1.S2. The intersection of the two-state model and the allosteric ternary complex model. The two models can be expressed together to demonstrate that they share a common ligand association step as illustrated above.

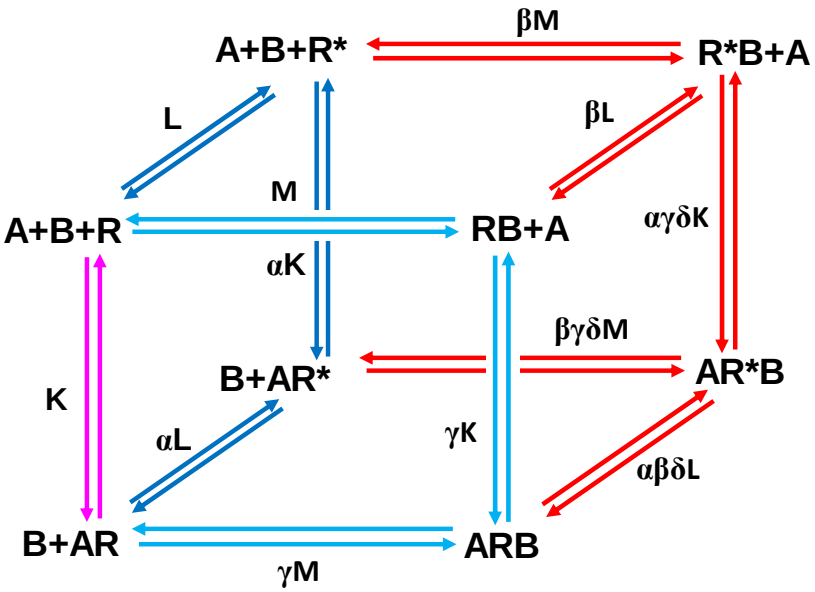


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Figure 1.S3. The allosteric two-state model. The complete allosteric two-state model includes the insertion of two additional active states which may exist in the presence of two ligands. Furthermore, it defines the cooperativity parameters which would connect these two additional states.

Chapter 2

Materials and Methods

2.1 Materials

[^{3}H]N-methylscopolamine (70-82 Ci/mmol) was purchased from Perkin Elmer/NEN (Boston, MA) and [^{3}H]myo-inositol (20 Ci/mmol) and [^{3}H]Arachidonic Acid (100 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). N-ethylamiodarone was obtained from IQSynthesis (Maryland Heights, MO). All other reagents were purchased from Sigma-Aldrich.

2.2 Cell culture

CHO cells stably transfected with human M_1, M_2, M_3, and M_5 receptors were used for all binding and response assays. Cells were grown in F-12 supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in 5% CO_2 and 100% humidity.

2.3 Membrane preparation

Membranes were collected by harvesting cells in ice cold 5 mM phosphate buffer (PB; 1 mM KH_2PO_4, 4 mM Na_2HPO_4, pH 7.4). Cells were homogenized on ice, with three 15 second pulses of a Bio Homogenizer from Biospec Products, Inc. (Bartlesville, OK) and centrifuged at 50,000g for 30 minutes. The supernatant was discarded and the pellet was resuspended in ice cold 5 mM PB and stored, in aliquots, at -70°C.

2.4 Radioligand binding assays

For binding experiments, amiodarone was dissolved in DMSO and the DMSO concentration was maintained below 1% for all assays; all other reagents were dissolved in buffer or deionized water. Equilibrium binding studies were performed as previously described (Ellis and Seidenberg, 1999 and Stahl and Ellis, 2010), except as noted. Briefly, these studies were performed in PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$,

pH 7.4, for 1 hour at 25°C. Binding was measured at 1 nM [^{3}H]NMS and non-specific binding was determined in the presence of 1 µM atropine. The affinity of NMS in this buffer is 0.204 nM for M_1 and 0.372 nM for M_5.

Dissociation rate binding studies were performed in PBS + 1 mM $CaCl_2$ and 1 mM $MgCl_2$ or 5 mM PB, as noted, and 1 nM [^{3}H]NMS at 25°C. Membranes were prelabeled with [^{3}H]NMS for 30 minutes, followed by addition of 3 µM atropine with or without increasing concentrations of the allosteric modulator(s). Assays were allowed to proceed for 20‑30 minutes (M_1) or 90‑120 minutes (M_5) and terminated by filtration.

Equilibrium and dissociation binding assays were terminated by rapid filtration through GF/B glass fiber filters (pretreated with 0.1% polyethyleneimine) on a Brandel cell harvester to trap membranes, and the filters were then rinsed twice with ice-cold 40 mM PB (pH 7.4). Bound radioactivity was measured by liquid scintillation in a Beckman LS6500 counter.

Intact cell binding was performed by incubating cells with 1 nM [^{3}H]NMS in PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4, for 30 minutes at 25°C. Nonspecific binding was determined with 3 µM atropine, and comprised approximately 1% of total binding. Following the incubation, cells were rinsed twice with PBS and solubilized in 1% SDS; the solubilized cells were transferred to scintillation vials and counted by liquid scintillation in a Beckman LS6500 counter.

2.5 Arachidonic acid release

Measurement of [^{3}H]AA release was adapted from the protocols of (Conklin et al., 1988 and Felder et al., 1990). CHO cells were seeded on 48-well plates (Greiner Bio-One, Germany) at a density of 29k cells/well in 0.25 mL F-12. Cells were

incubated until they attached (approximately 3 hours) and the media was exchanged for media containing 0.025 µCi [^{3}H]AA per well. The cells were then grown for 16-20 hours before the assay was performed. [^{3}H]AA release was measured in Eagle's Basal Medium + 2 mg/mL fatty acid free bovine serum albumin (EM-BSA). Where indicated, studies were performed in bicarbonate-buffered EM-BSA. However, most experiments were performed in EM-BSA buffered with 20 mM HEPES. Cells were rinsed twice with EM-BSA, followed by addition of EM-BSA media containing experimental agents (concentrated stock solutions of all experimental agents were prepared in deionized water). Cells were then incubated for 1 hour at 37°C. The assay was terminated by aspiration of the media and the amount of radioactivity collected was determined by liquid scintillation in a Beckman LS6500 counter.

2.6 Inositol phosphate metabolism

The protocol used for measuring inositol phosphate (IP) metabolism was adapted from Ellis et al. (1990) and Bymaster et al. (1999). Briefly, cells were cultured in 24-well plates (BD Falcon, San Jose) at a density of 50k cells/well in 0.5 mL F-12. Cells were incubated until they attached and the medium was exchanged for medium containing 2 µCi [^{3}H]myo-inositol per well followed by overnight incubation. The following day, cells were rinsed and incubated in Eagle's Basal Medium with 20 mM HEPES and 10 mM LiCl for 15 minutes. Following the preincubation, Eagle's Basal Medium with 20 mM HEPES and 10 mM LiCl and the appropriate experimental agents was added (concentrated stock solutions of all experimental agents were prepared in deionized water) and the assay was allowed to proceed for 30 minutes at 37°C. After 30 minutes, the cells were placed on ice, the medium was removed, and 0.5 mL ice-cold methanol

was added and allowed to sit for 5 minutes. Then 0.5 mL ice-cold H_20 was added to each sample. Prior to application of the sample, Dowex columns (100-200 mesh) were treated with 3 M ammonium formate in 0.1 M formic acid and rinsed with 0.1 M formic acid. A portion of each sample (0.8 mL) was transferred to a column, after which the columns were rinsed with 0.1 M formic acid and eluted into scintillation vials with 0.8 M ammonium formate in 0.1 M formic acid. The amount of radioactivity collected was determined by liquid scintillation in a Beckman LS6500 counter.

2.7 Receptor alkylation

Where noted, receptor number was reduced by treatment with the irreversible antagonist phenoxybenzamine (POB). The protocol was similar to that used by Leff et al. (1993). Specifically, POB was dissolved in ethanol and stored at -20°C. It was diluted in Eagle's Basal Medium with 20 mM HEPES immediately before use and the ethanol concentration was maintained below 1%. Cells were incubated in medium containing POB for 30 minutes at 37°C. Following incubation, two quick washes were performed followed by three 5 minute washes. All washes were in Eagle's Basal Medium with 20 mM HEPES. In the case of measuring IP metabolism, the three 5 minute washes were performed in the presence of 10 mM LiCl. After the washes, intact cell NMS binding, AA release, and IP metabolism were measured in the manner described above.

2.8 Data analysis

2.8.1 Binding and response analysis

Response curves, and some binding curves, were fit to an empirical four-parameter equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{n(\log C_{50} - X)}} \qquad \text{[eq. 1]}$$

where X is the log of the concentration of the ligand used, Y is the amount of binding or response, C_{50} is the concentration of the ligand that produces 50% of the maximal effect, Top and $Bottom$ are the top and bottom plateaus of the curve, and n is related to the Hill slope for the curve. In order to determine significance in changes of potency and maximal response between curves, the curve-fits were evaluated by F-test, using Prism (Graphpad Software, Inc., San Diego). The stated parameter values were derived by computer generated curve-fitting estimates of mean and S.E.M. Where indicated, a t-test was used to determine significance ($p < 0.05$), based on calculated means and S.E.M. values.

2.8.2 Analysis of allosteric ligand affinity and cooperativity

In order to determine affinity values for the allosteric ligand and cooperativity values present between the orthosteric and allosteric ligands, data from equilibrium binding studies was fit to the allosteric ternary complex model (modified from Ehlert, 1988):

$$Y = \frac{[X][B_{\max}]}{[X] + K_X\left(\frac{K_A + [A]}{K_A + \alpha[A]}\right)} \qquad \text{[eq. 2]}$$

where X and A represent the concentrations of the orthosteric and allosteric ligands used, respectively, Y is the amount of binding, K_X and K_A refer to the dissociation constants for each ligand, B_{max} is the value for saturation binding, and α is the binding cooperativity exhibited between the two ligands. In this formulation, $\alpha < 1$ is indicative of negative cooperativity.

2.8.3 Analysis of allosteric modulation of off-rate

The analysis of experiments on the dissociation of [^{3}H]NMS required that the data be expressed in a time-independent manner. This was accomplished by fitting the time-dependent loss of binding to a mono-exponential function to determine the rate constant of dissociation. These rate constants were then fit to the following equation:

$$\frac{k_{obs}}{k_0} = 1 - \frac{mL}{L + K} \qquad \text{[eq. 3]}$$

where k_{obs} is the rate constant observed in the presence of a given concentration of the allosteric ligand and k_0 is the rate constant in the absence of allosteric ligand, L is the concentration of the allosteric ligand, m defines the maximal effect of the allosteric ligand on the rate of dissociation of NMS, and K represents the equilibrium dissociation constant for the interaction of the allosteric ligand with the NMS-bound form of the receptor. In some cases, data from equilibrium and dissociation assays were fit simultaneously, to evaluate the consistency of the model; when this was done, K ([eq. 3]) was constrained to be equal to αK_A ([eq. 2]), and the effect on the goodness of fit was evaluated by an F-test, using the curve-fitting program Prism (Graphpad Software, Inc., San Diego).

2.8.4 Predicted competition at the allosteric site in off-rate studies

To test whether two allosteric ligands, L_1 and L_2, interacted competitively at a single allosteric site, the parameters for both ligands, obtained from analyses of separate experiments according to [eq. 3], were used to simulate the expected competitive behavior, according to the following equation (adapted from Ellis and Seidenberg, 1992):

$$\frac{k_{obs}}{k_0} = 1 - \frac{m_1 L_1}{L_1 + K_1(1 + \frac{L_2}{K_2})} - \frac{m_2 L_2}{L_2 + K_2(1 + \frac{L_1}{K_1})} \qquad \text{[eq. 4]}$$

2.8.5 Analysis of receptor reserve

Confirmation of the presence of a receptor reserve depends on the demonstration that the ability to produce a response is disproportionately preserved, relative to the loss of receptors. When the receptor reserve is very large, the initial effect of irreversible antagonism is to shift the response curve to the right with negligible loss of maximal response. As greater numbers of receptors are inactivated, the maximal response is attenuated and the EC_{50} typically approaches the K_A value of the agonist (see Furchgott, 1955).

This data can be analyzed with an equation that assumes that both binding and response are adequately represented by simple hyperbolic functions. If A is the concentration of agonist and the fractional response, Y, is represented by $A/(A+EC_{50})$, while fractional occupancy, X, is represented by $A/(A+K_A)$, then Y can be represented as a function of X by the equation:

$$Y = \frac{X}{X(1-r) + r} \qquad \text{[eq. 5]}$$

where $r = EC_{50}/K_A$. When $r = 1$, response is directly proportional to the number of binding sites remaining; the presence of a receptor reserve corresponds to r values that are significantly less than one.

2.8.6 Response modeling with the four-ligand allosteric two-state model

The 4L-ATSM, presented in Figure 4.1 (p. 71), was employed in response simulations. The parameters used to construct the model are defined in Table 2.1. These parameters represent specific receptor or ligand constants: equilibrium association constants of ligands for their respective sites on the receptor (K_1, K_2, M_1, and M_2); intrinsic efficacy values corresponding to a ligand's ability to stabilize the active state of the receptor (α_1, α_2, β_1, and β_2,); the receptor isomerization constant (L); and parameters which describe how ligands cooperatively modulate each other's effects on binding or activation (γ_{11}, γ_{12}, γ_{21}, γ_{22}, δ_{11}, δ_{12}, δ_{21}, and δ_{22}). The parameter values used to generate the simulations presented in Figure 4.9 are stated in Table 4.2 (p. 89). The equation defines response as the ratio of receptors in the active conformation to the total receptor number, R_{active} / R_{total} (elaborated on in *Section 4.4*). In the simulations based on the model, the parameters focused on are the γ and δ parameters. The other parameters are set to values that are reasonable and convenient. For example, K_1 and M_1 are arbitrarily set to 1; L is set to 0.01 to indicate negligible basal activity; α_1 is set to 100 to indicate robust agonist activity that can be modulated up or down. β_1 is set to 1, to indicate that the allosteric ligand possesses no agonist activity by itself (in agreement with amiodarone's observed properties).

Table 2.1. Definition of parameters for the four-ligand allosteric two-state model

Parameter Name	Parameter Definition
A_1	[Orthosteric ligand 1]
A_2	[Orthosteric ligand 2]
B_1	[Allosteric ligand 1]
B_2	[Allosteric ligand 2]
K_1	Association constant of A_1
K_2	Association constant of A_2
L	Receptor isomerization constant
M_1	Association constant of B_1
M_2	Association constant of B_2
α_1	Intrinsic efficacy of A_1
α_2	Intrinsic efficacy of A_2
β_1	Intrinsic efficacy of B_1
β_2	Intrinsic efficacy of B_2
γ_{11}	Binding cooperativity between A_1 and B_1
γ_{12}	Binding cooperativity between A_1 and B_2
γ_{21}	Binding cooperativity between A_2 and B_1
γ_{22}	Binding cooperativity between A_2 and B_2
δ_{11}	Activation cooperativity between A_1 and B_1
δ_{12}	Activation cooperativity between A_1 and B_2
δ_{21}	Activation cooperativity between A_2 and B_1
δ_{22}	Activation cooperativity between A_2 and B_2

Chapter 3

Novel allosteric effects of amiodarone at the muscarinic M_5 receptor

3.1 Introduction

Muscarinic receptors are expressed throughout the central nervous system and have been implicated in numerous neurological disorders including Alzheimer's disease, Parkinson's disease, schizophrenia, and addiction (Ellis, 2002; Wess et al., 2007; Langmead et al., 2008b). Five subtypes of muscarinic receptors exist (M_1-M_5), all of which are G protein-coupled receptors and share acetylcholine (ACh) as their endogenous neurotransmitter. To a first approximation, the muscarinic receptors can be separated into two classes. The M_2 and M_4 receptors inhibit adenylate cyclase by activating G_i. The M_1, M_3, and M_5 receptors stimulate lipid metabolism via G_q-mediated activation of phospholipase C, which then leads to mobilization of intracellular calcium. Ultimately, these intracellular muscarinic signaling responses are much more detailed and complex, and include activation of protein kinases (including MAP kinases), activation of phospholipases A_2 and D (releasing arachidonic acid and choline, respectively), and modulation of potassium and calcium channels (Lanzafame et al., 2003). The advent of molecular cloning and expression has provided the ability to characterize specific muscarinic subtypes with regard to their involvement in various signaling pathways. However, it has proven to be difficult to connect each of these subtypes with their physiological role due to a lack of small-molecule ligands that are highly subtype selective. The most precise documentation of the physiological involvement of specific subtypes has been obtained through the evaluation of knock-out mice. For example, in such studies, the lack of the M_1 receptor has been connected to specific deficits in working memory and consolidation (Anagnostaras et al., 2003), whereas M_5 has been found to be important in other cognitive tasks, perhaps due to its

role in regulating the dilation of cerebral blood vessels (Yamada et al., 2001; Araya et al., 2006).

At the molecular level, the muscarinic receptors exhibit an unusually high degree of sequence homology at the orthosteric acetylcholine binding site, a property that has hindered the development of subtype selective ligands (Jones et al., 1992). Many agonists and antagonists bind to the muscarinic orthosteric site with excellent affinity, but none of these is highly subtype selective. This lack of subtype selectivity means that compounds with high affinity for the muscarinic family have undesirable side effects, caused by interactions with multiple subtypes in the family, and this has limited their therapeutic utility. For these receptors to be successfully targeted clinically, ligands with much better subtype selectivity will be necessary.

The failure to develop ligands that target the orthosteric site of individual subtypes has led many investigators to pursue ligands that target allosteric sites. An allosteric site is defined as a binding site, physically distinct from the orthosteric agonist binding site, that may influence the binding properties of the orthosteric site (Christopoulos and Kenakin, 2002). All subtypes of muscarinic receptors are known to possess allosteric sites (Ellis et al., 1991), and allosteric modulators present several possible advantages over orthosteric agonists and antagonists. Specifically, allosteric modulators may have greater binding selectivity, if they are able to bind to less conserved regions of the receptor. Even if they lack binding selectivity, they may be selective on the basis of the degree of cooperativity they possess. That is, an allosteric ligand may be positively cooperative with the endogenous ligand at one receptor subtype, but neutral at all of the other subtypes. Lazareno et al. (1998) called this type

of action “absolute selectivity”. Furthermore, and especially important in the CNS, positive allosteric modulators that exert no effect by themselves will only amplify the response of a particular receptor subtype when the endogenous transmitter is present, thus preserving the physiological spatiotemporal patterning of synaptic transmission (Ellis, 1997**;** Conn et al., 2009).

The commonly used antiarrythmic drug amiodarone has been reported to interact with numerous physiological targets. Studies have shown that amiodarone interacts with several ion channels, inhibiting sodium, calcium, and potassium currents (Kodama et al., 1997) and alters adrenergic receptor signaling (Schnabel et al., 1999; Yin et al., 1994). Amiodarone has also been found to inhibit the binding of antagonists to muscarinic receptors (Cohen-Armon et al., 1984; Colvin et al., 1989).

The findings presented in this chapter are from binding studies and functional assays, at M_1 and M_5 muscarinic receptors, specifically designed to distinguish allosteric or competitive interactions. We have established that amiodarone binds at an allosteric site on M_1 and M_5 receptors, but that this allosteric site differs from another allosteric site that binds gallamine, brucine, and many other muscarinic allosteric ligands. In functional assays, amiodarone enhances the response to ACh at M_5 receptors, but not at M_1 receptors.

3.2 Results

3.2.1 Amiodarone allosterically modulates [^{3}H]NMS binding properties

In agreement with previous studies (Cohen-Armon et al., 1984; Colvin et al., 1989), amiodarone was found to inhibit antagonist binding at muscarinic receptors. Figures 3.1A and 3.1B show that the binding of the orthosteric antagonist [^{3}H]NMS was inhibited by micromolar concentrations of amiodarone at both M_1 and M_5 receptors These binding studies were performed in a physiological buffer, PBS + 1mM $CaCl_2$ and 1mM $MgCl_2$. Under these conditions, amiodarone was not able to completely inhibit the specific binding of [^{3}H]NMS at either subtype. That is, the bottom plateau was significantly different from zero [M_1, $F(1, 17) = 31.96$, $p < 0.0001$; M_5, $F(1, 17) = 58.65$, $p < 0.0001$]. Amiodarone inhibited 82% of specific binding at the M_1 receptor, with a $\log IC_{50}$ of -6.11 ± 0.13, and 64% of specific binding at the M_5 receptor, with a $\log IC_{50}$ of -5.47 ± 0.04. This finding suggests that amiodarone and NMS bind to different sites, such that both ligands can bind to the receptor simultaneously at sufficiently high concentrations. By comparison, Figure 3.1C shows equilibrium inhibition curves for atropine and ACh under identical conditions at the M_1 receptor. Atropine and ACh completely inhibited the specific binding of [^{3}H]NMS, as would be expected for these competitive ligands, with $\log IC_{50}$ values of -8.28 ± 0.08 and -4.66 ± 0.06, respectively.

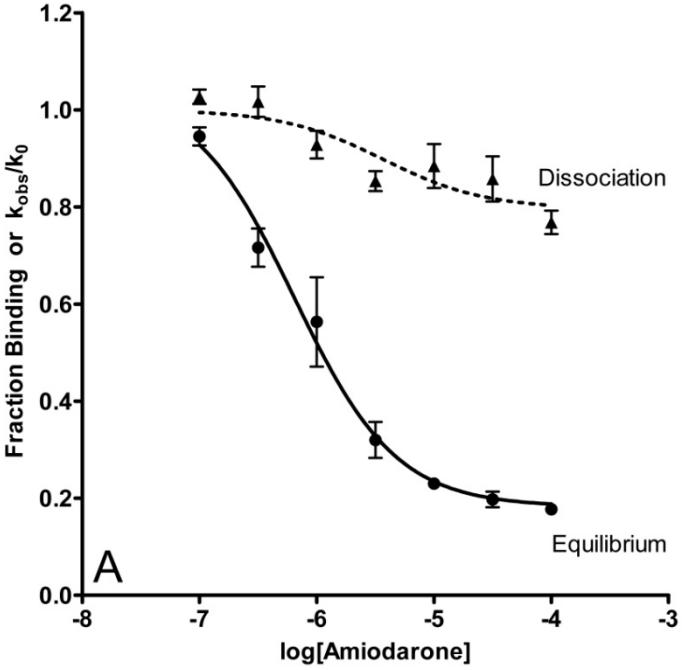
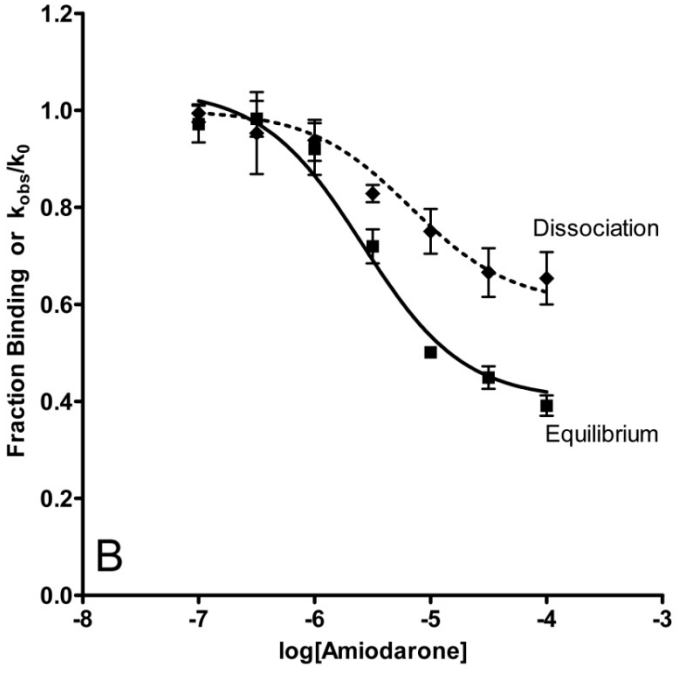
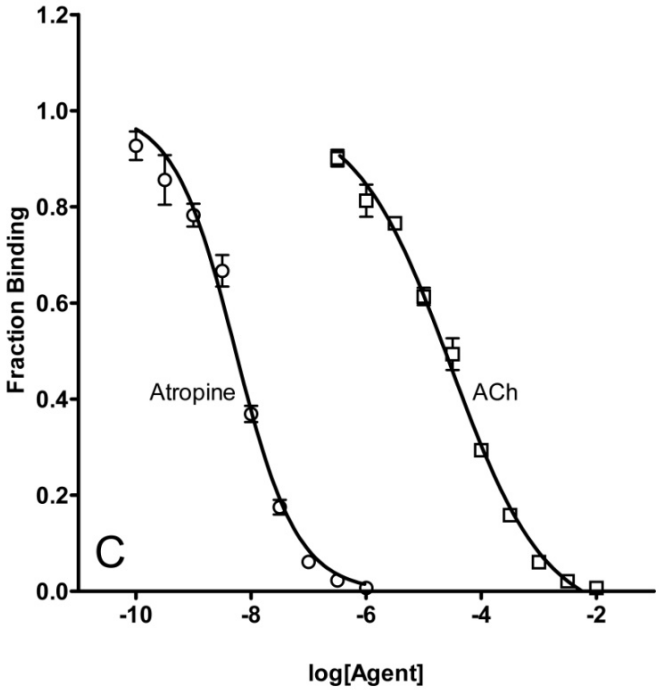


Figure 3.1. [^{3}H]NMS binding and dissocation is inhibited by amiodarone. Amiodarone inhibited equilibrium binding and slowed the rate of [^{3}H]NMS dissociation at both M_1 and M_5 receptors, in a concentration-dependent manner. The effect of amiodarone on equilibrium binding is shown at M_1 (Panel A: ●) and M_5 (Panel B: ■). Binding inhibition was incomplete at both subtypes. Amiodarone's effect on the dissociation rate is shown at M_1 (Panel A: ▲) and M_5 (Panel B: ♦). The two sets of data in each panel were simultaneously fit under the constraint that they accommodate the allosteric ternary complex model (see text for statistical analysis and best-fit values of each curve). This constrained fit is represented by solid lines (equilibrium) and dotted lines (dissociation). Panel C shows the inhibition of [^{3}H]NMS binding, at M_1, by the competitive ligands atropine(○) and ACh(□). Curves are the best fits to [eq. 3]. Each data point is the average of three experiments expressed as mean ± SEM.





One of the signature features of allosteric interactions is the ability of one ligand to alter the rate of dissociation of another ligand (Ellis, 1997; Ellis et al., 1991). To investigate this possibility, M_1 and M_5 receptors were prelabeled with [^{3}H]NMS and the rate of dissociation was monitored in the absence or presence of amiodarone. It was established that dissociation followed a mono-exponential decline in both the presence and absence of amiodarone (Figure 3.S1; p. 65) and subsequent determinations were based on "two-point" assays (Ellis et al., 1991; Kostenis and Mohr, 1996). The dissociation rates (k_{off}) in the absence of amiodarone were found to be 0.0640 min^{-1} for M_1 and 0.0149 min^{-1} for M_5. Amiodarone was able to slow the rate of dissociation of [^{3}H]NMS to a maximal degree of between 20-40% at both receptor subtypes, with apparent log affinities of -5.84 ± 0.13 at M_1 and -5.50 ± 0.04 at M_5 (Figures 3.1A and 3.1B).

The equilibrium and dissociation studies described above were carried out in the same buffer, so they can be directly compared. According to the allosteric ternary complex model, the interaction of the allosteric ligand with the receptor will alter the affinity of the orthosteric ligand. The change in the affinity of the orthosteric ligand caused by the allosteric ligand is the cooperativity factor, α. The model predicts the IC_{50} of amiodarone in dissociation studies should be the same as the product of K_A and α, determined at equilibrium. The results from the equilibrium and dissociation studies were analyzed using simultaneous curve fitting with equilibrium data fit to [eq. 2] and dissociation data fit to [eq. 3], as described in *Section 2.8.3*. When the IC_{50} was allowed to float independently of αK_A, the fit of the combined data to the two equations was not significantly better than when they were constrained to be equal. That is, the IC_{50} was

not significantly different from the αK_A, for either receptor subtype [M_1, $F(1,36) = 0.3849$, $p = 0.5389$; M_5, $F(1,43) = 3.405$, $p = 0.0719$]. This indicates that the equilibrium and dissociation data are consistent with each other and with the model. The best-fit values for the constrained analysis are $\log K_A = -6.9$ and $\alpha = 0.03$, for M_1 and $\log K_A = -6.0$ and $\alpha = 0.14$, for M_5.

3.2.2 Amiodarone interacts with a novel allosteric site

When two allosteric modulators exert markedly different maximal effects on the rate of dissociation of the orthosteric ligand, it is possible to test whether they interact at a common site. This technique has previously been used to demonstrate that gallamine and obidoxime appear to compete for a common allosteric site on the M_2 muscarinic receptor (Ellis and Seidenberg, 1992). Data from the former paper has been redrawn in Figure 3.2C as an example of competition at the allosteric site. In order to investigate the location at which amiodarone exerts its effects, these binding studies were performed in 5mM PB. The affinity of amiodarone is much higher in this low ionic-strength buffer, a phenomenon that has been reported for many muscarinic allosteric ligands (e.g., Trankle et al., 1996; Ellis et al., 1991). Under these conditions, amiodarone slowed the rate of [^{3}H]NMS dissociation by approximately 40% at both receptor subtypes, with apparent log affinities of -7.25 ± 0.09 at M_1 and -7.24 ± 0.42 at M_5 (Figures 3.2A and 3.2B). The dissociation rates (k_{off}) in the absence of amiodarone were found to be 0.0494 min^{-1} for M_1 and 0.0122 min^{-1} for M_5, in good agreement with previous studies in this buffer (Ellis et al., 1991). Gallamine, another well-known muscarinic allosteric ligand, was able to slow the dissociation of [^{3}H]NMS to a significantly greater degree at both receptors, reaching a 78% reduction at M_5 with an

apparent log affinity value of −5.06 ± 0.13 and a 97% reduction at M_1 with an apparent log affinity of −5.70 ± 0.03. It can be seen in Figure 3.2C that obidoxime partially reverses the effect of gallamine on the rate of dissociation of [^{3}H]NMS (solid squares), and that the concentration-dependence of that reversal is in excellent agreement with the predictions of the model of competitive interaction at an allosteric site (dashed line). This suggests that gallamine and obidoxime bind to the same physical site (although it also remains possible that the binding of the two ligands reflects a strong negative cooperativity). On the other hand, when analogous experiments were conducted with gallamine and amiodarone at the M_1 and M_5 receptors, amiodarone was not able to significantly reverse the effects of gallamine and the data deviated dramatically from the predictions of the model (indicated by the dashed lines in Figures 3.2A and 3.2B). Thus, it is clear that amiodarone and gallamine must be acting at physically distinct allosteric sites.

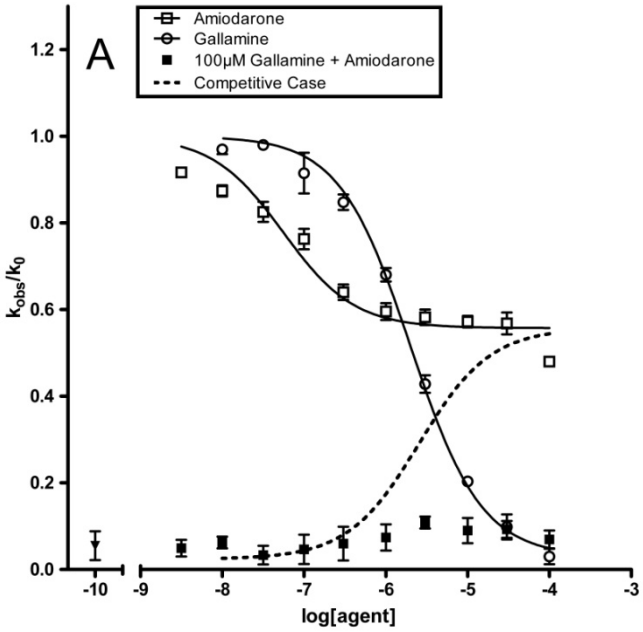
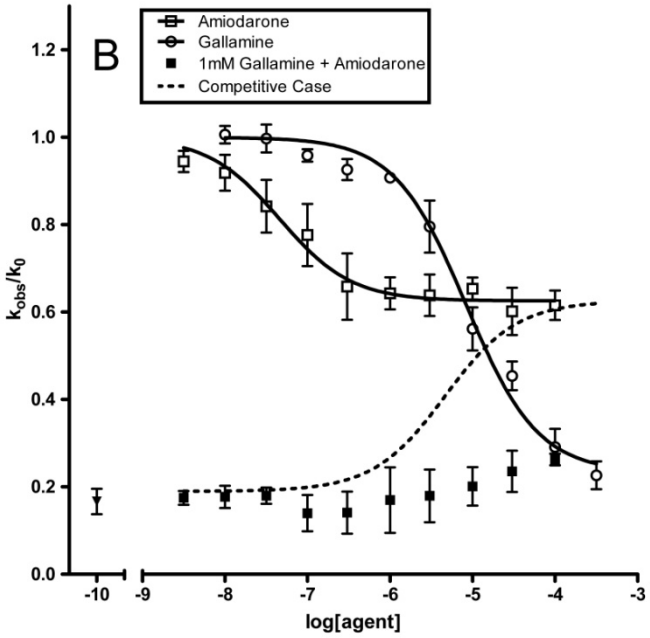
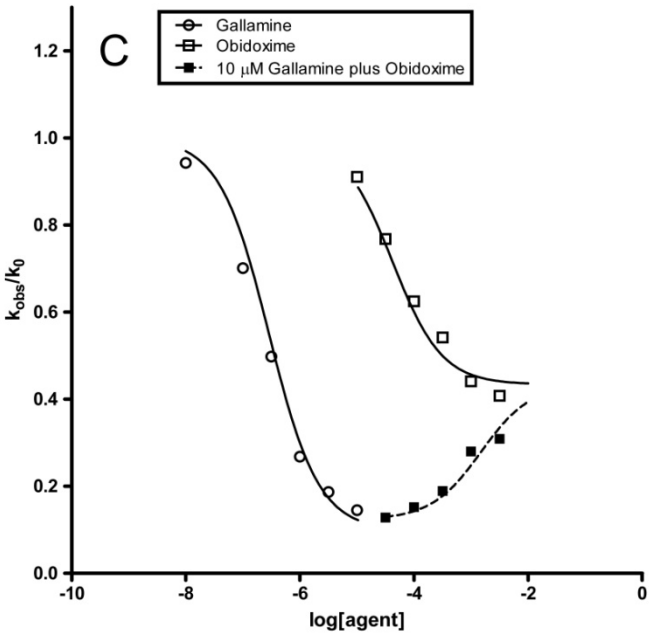


Figure 3.2. Amiodarone and gallamine do not act at a common allosteric site. Amiodarone and gallamine both slow the dissociation of [^{3}H]NMS from M_1 (Panel A) and M_5 (Panel B). Simulation of the data that would be expected of a competitive interaction between amiodarone and gallamine is presented as the dotted line, in each panel, according to [eq. 4]. At both subtypes, it is clear that amiodarone was not able to reverse the inhibitory effects of gallamine in the manner predicted by the competitive model. Solid lines are the best fit to [eq. 3]. Each point is the average of three experiments expressed as mean ± SEM. Panel C is redrawn from Ellis and Seidenberg (1992) and indicates that gallamine and obidoxime are competitive at a common site. The best-fit values according to [eq. 4] were:
Panel A (M_1): amiodarone, m=0.443, pK=7.25; gallamine, m=0.970, pK=5.70.
Panel B (M_5): amiodarone, m=0.388, pK=7.24; gallamine, m=0.775, pK=5.06.
Panel C (M_2): obidoxime, m=0.566, pK=4.38; gallamine, m=0.902, pK=6.54.





3.2.3 Amiodarone enhances agonist-induced response at M_5, but not M_1, receptors

Initial experiments indicated that acetylcholine stimulated the release of [^{3}H]AA from CHO cells that stably express M_1 or M_5 receptors. These experiments were conducted in a bicarbonate-buffered system, as described in *Section 2.5*, and established that EC_{20} values for acetylcholine were approximately 30 nM for M_1 and 3 nM for M_5. In the presence of 3 nM acetylcholine, the addition of amiodarone resulted in a concentration-dependent increase in M_5 mediated response (Figure 3.3A); the response elicited by 3 nM acetylcholine in the presence of the highest concentration of amiodarone was approximately equal to the response produced by 1 mM acetylcholine alone. However, over the same concentration range of amiodarone, there was no significant enhancement of the acetylcholine-induced response from M_1-expressing CHO cells (Figure 3.3B). Amiodarone also had no effect on basal response in the concentration range examined (data not shown).

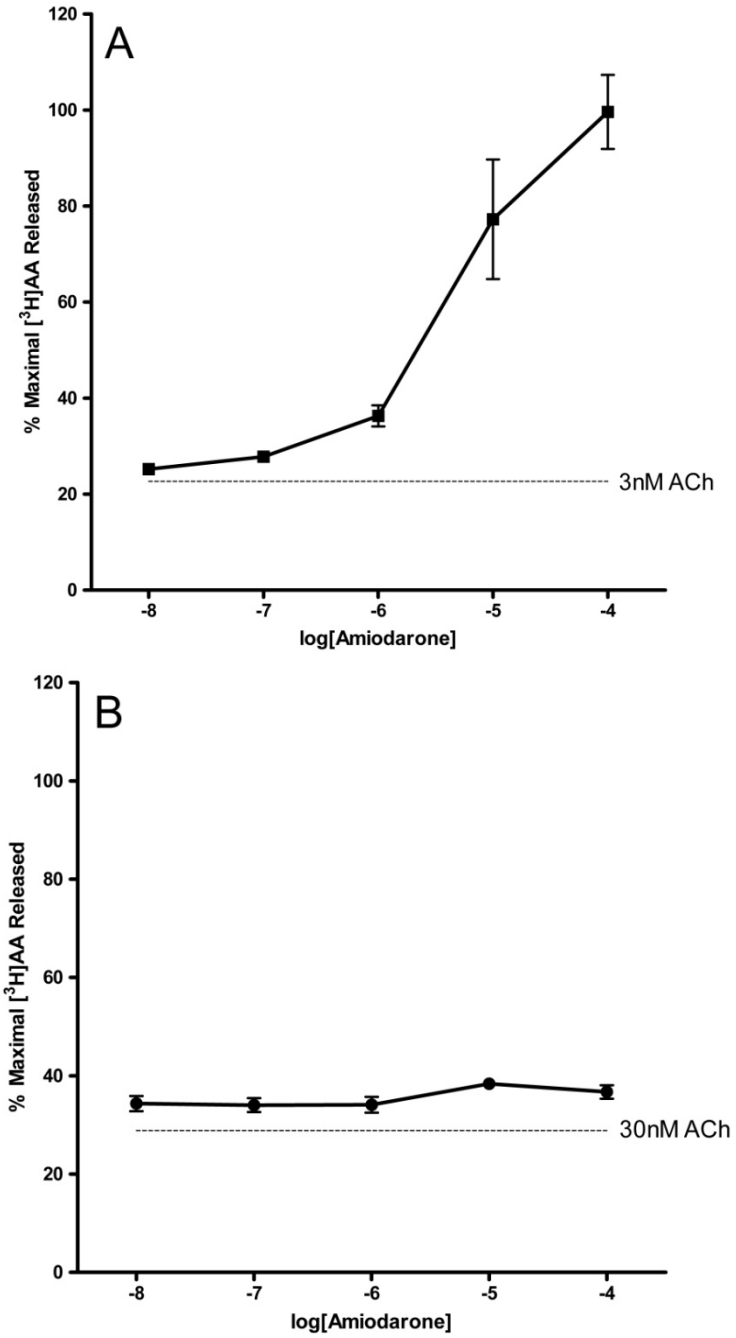


Figure 3.3. Subtype selective enhancement of ACh stimulated [^{3}H]AA release by amiodarone. CHO-M_5 cells (Panel A) and CHO-M_1 cells (Panel B) were exposed to ACh at EC_{20} concentrations (3 nM and 30 nM, respectively) with increasing concentrations of amiodarone. Data are expressed as a percentage of the response generated by saturating concentrations of ACh (1 mM). This is a representative experiment, carried out in bicarbonate-buffered medium.

In subsequent studies, the ability of amiodarone to modulate the responses of a series of muscarinic agonists at the M_1 and M_5 receptors was investigated. These experiments were carried out in a HEPES-buffered system, as described in *Section 2.5*. In order to choose appropriate agonist concentrations to test with amiodarone, response curves were generated for each of the four agonists (acetylcholine, oxotremorine-M, oxotremorine, and pilocarpine); the results of these experiments are summarized in Table 3.1. It is notable that the potency of acetylcholine is slightly higher toward the M_5 receptor in the bicarbonate system and much higher (approximately 25-fold) toward M_1 (judging by the responses to acetylcholine alone in bicarbonate, Figure 3.3, relative to the parameters obtained in HEPES, Table 3.1). As has been observed by other investigators (Bymaster et al., 1999), oxotremorine and pilocarpine were partial agonists, compared to acetylcholine. Oxotremorine-M exhibited a slightly higher E_{max} than acetylcholine. All of the agonists displayed significantly higher potency at the M_5 receptor than at the M_1 receptor. The data in Table 3.1 was used to select the concentration of each agonist that would yield a response in the range of 20% to 40% of the maximal effect, and the ability of 30 μM amiodarone to modify those responses was tested. The results were consistent with those described above for acetylcholine. At the M_5 receptor, the response of each agonist was significantly enhanced by amiodarone, while the responses at the M_1 receptor were not significantly affected (Figure 3.4).

Table 3.1. Response parameters of multiple muscarinic agonists.
Data is the average of two or three experiments (mean ± SEM). Experiments were performed in quadruplicate and maximal response is expressed as a percentage of [^{3}H]AA release stimulated by 1 mM ACh.

CHO-M_1	**logEC_{50}**	**E_{max} (% max)**
Acetylcholine	-5.8 ± 0.04	100
Oxotremorine-M	-6.4 ± 0.03	113 ± 4.2
Oxotremorine	-6.9 ± 0.02	62 ± 1.2
Pilocarpine	-5.7 ± 0.0	52 ± 1.3
CHO-M_5		
Acetylcholine	-7.6 ± 0.07	100
Oxotremorine-M	-7.6 ± 0.11	105 ± 4.2
Oxotremorine	-7.7 ± 0.15	72 ± 2.5
Pilocarpine	-6.1 ± 0.06	59 ± 1.2

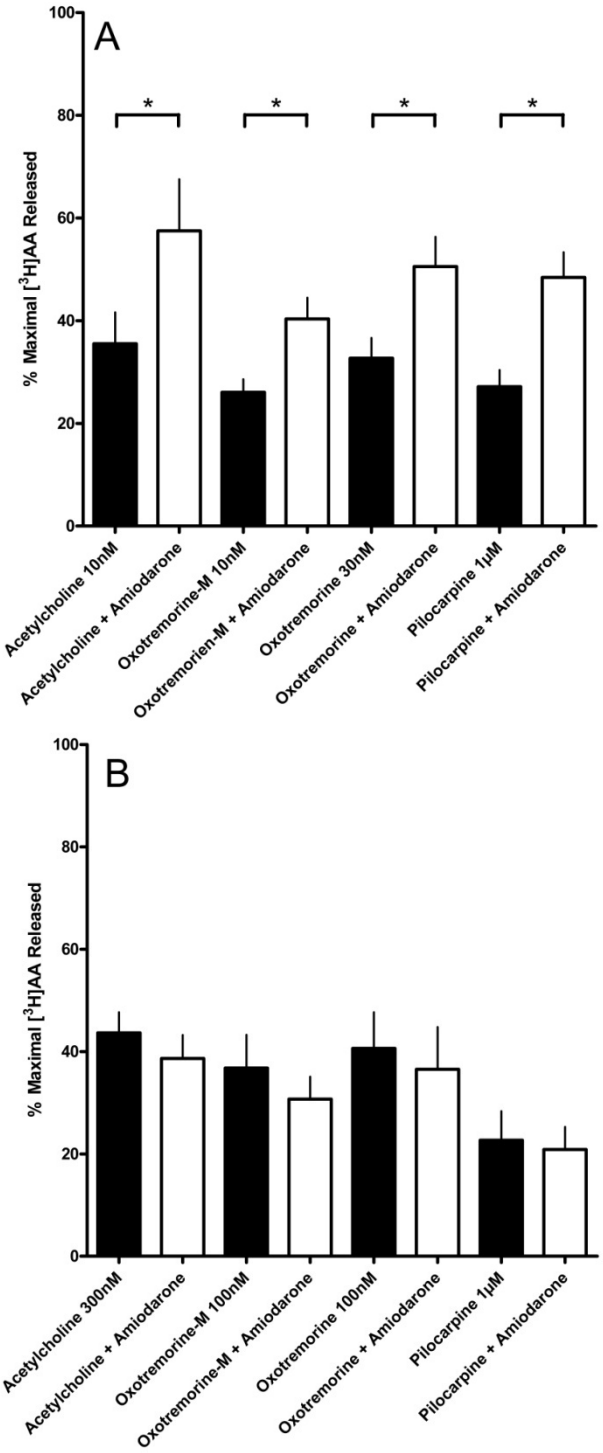


Figure 3.4. Effect of amiodarone on the stimulation of [^{3}H]AA release by multiple agonists. CHO-M_5 cells (Panel A) and CHO-M_1 cells (Panel B) were incubated with the agonist concentrations indicated, with or without 30 μM amiodarone. Amiodarone enhanced the responses of all of the agonists at M_5, but did not significantly affect the M_1 mediated responses. Curves show data normalized to maximal [^{3}H]AA release generated by 1 mM ACh. Each bar is the average of three experiments expressed as mean ± SEM. *$p < 0.05$

3.2.4 Amiodarone enhances efficacy (not potency) of acetylcholine at the M_5 receptor

More detailed studies were carried out with M_5-expressing CHO cells to investigate the mechanism by which amiodarone enhances acetylcholine-induced response. As in Figure 3.4 and Table 3.1, these studies were conducted in HEPES buffered medium (see *Section 2.5*). Somewhat surprisingly, acetylcholine's $logEC_{50}$ was inhibited slightly in the presence of amiodarone, from -7.65 ± 0.07 to -7.45 ± 0.09, although this effect did not achieve significance [$F(1, 56) = 1.841$, $p = 0.1803$]. Rather, it was the maximal response elicited by acetylcholine that was found to be enhanced by the presence of 30 μM amiodarone (Figure 3.5). This effect was highly significant [$F(1, 56) = 108.3$, $p < 0.0001$].

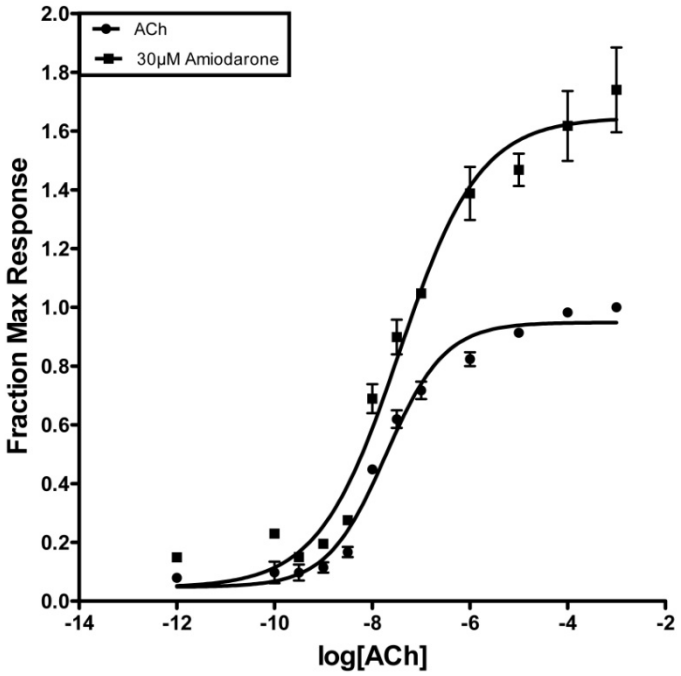


Figure 3.5. Effect of amiodarone on ACh concentration-response curves at the M_5 receptor. Amiodarone enhances the maximal effect elicited by ACh, without enhancing the potency of the agonist. Data were normalized to the degree of [^{3}H]AA release generated by 1 mM ACh. Curves are the best fits to [eq. 1]; see text for parameter values and statistics. Each point is the average of three experiments expressed as mean ± SEM.

3.3 Discussion

Two previous studies have found that amiodarone interacts with muscarinic receptors and inhibits radioligand binding (Cohen-Armon et al., 1984; Colvin et al., 1989). They concluded that amiodarone inhibited the binding of orthosteric muscarinic antagonists in a competitive manner in most or all of the preparations investigated. However, their studies differed from our studies in that they used different labeled ligands and different receptors in their binding assays; one study used canine heart membranes (i.e., M_2), while the other used rat heart (M_2) or brain membrane homogenate (mixed subtypes). Either one of these differences could be responsible for some divergence from our findings, as the choice of orthosteric ligand and receptor subtype are both known to affect the degree of cooperativity observed by an allosteric ligand. Even so, both papers noted some characteristics of their binding assays that deviated from competitive behavior. Cohen-Armon et al. noted that *"The existence of an allosteric site"* might explain anomalous binding behavior that was observed with brain stem membranes. Colvin et al. observed incomplete inhibition of radioligand binding by amiodarone that is similar to the data presented in Figure 3.1. Partial inhibition of radioligand binding could be indicative of receptor heterogeneity in a tissue that expresses multiple receptor subtypes. However, in a recombinant system that expresses only one receptor subtype, partial inhibition is not consistent with the competitive model; rather, it is definitive evidence that amiodarone does not interact with the orthosteric site, but only at an allosteric site on the receptor (Christopoulos and Kenakin, 2002). Under the conditions used in Figure 3.1, it is clear that amiodarone is only able to partially inhibit the equilibrium binding of [^{3}H]NMS at both M_1 and M_5

receptors. The allosteric nature of the interaction between amiodarone and muscarinic receptors was further confirmed by studies of radioligand binding kinetics. Amiodarone was found to slow the rate of [^{3}H]NMS dissociation from both M_1 and M_5 receptors to highly significant extents and with potencies that were in good agreement with the parameters of the equilibrium studies. Thus, amiodarone must be interacting with a region distinct from the ACh binding site, because orthosteric ligands do not change each other's rates of dissociation (Ellis, 1997).

It is important to examine statements made by Cohen-Armon et al. (1984) suggesting that amiodarone might have decreased the total number of binding sites (B_{max}) in binding studies of cardiac membranes (notably, they did not observe effects on B_{max} in membranes from forebrain regions, which would contain M_1 and possibly M_5 receptors). Curiously, these authors then elaborate on this point by further stating that "the inhibitory effect of amiodarone could be eliminated by consecutive washings", a finding that indicates that amiodarone does not act irreversibly and that thereby contradicts the suggested B_{max} effect. In subsequent studies, Colvin et al. (1989) did not report any reduction in B_{max} in any of their binding studies with cardiac membranes; furthermore we have not found any reduction in B_{max} in our own studies of M_2 receptors (Figure 3.S2; p. 66). Nevertheless, we have carried out additional binding studies with M_1, M_2, and M_5 receptors that have demonstrated that the effects of amiodarone are reversed by simple dilution (Figure 3.S3; p. 67). Thus, it does not appear that changes in B_{max} could have compromised any of our results.

Functional muscarinic receptor response was measured as the stimulation of [^{3}H]AA release from receptor-expressing CHO cells exposed to muscarinic agonists.

This assay was previously found to provide robust and reproducible results in multiple cell lines expressing muscarinic receptors, including CHO cells (Conklin et al., 1988; Bymaster et al., 1999). Amiodarone markedly enhanced the response of submaximal concentrations of ACh (EC_{20}) in cells expressing the M_5 receptor, at concentrations that were without effect in the absence of ACh; furthermore, amiodarone did not enhance the response of submaximal concentrations of ACh in cells expressing M_1 receptors (Figure 3.3). These results are, again, consistent with an allosteric site of action and suggest that amiodarone is a selective positive allosteric modulator (PAM) of muscarinic receptors. The action of amiodarone at the M_5 receptor was further investigated by evaluating the effect of a moderately high concentration of the allosteric modulator on the ACh concentration-response curve. Interestingly, the potency of ACh was not enhanced by amiodarone; rather, the maximal response to ACh was found to be significantly greater in the presence of 30 μM amiodarone (Figure 3.5). This finding runs counter to the common observation of PAM action at muscarinic receptors, which has typically been expressed as enhancement of the potency of muscarinic agonists. The first reported muscarinic PAM was brucine, which selectively enhanced the affinity of ACh for the M_1 subtype (Lazareno et al., 1998). Subsequently, a series of brucine analogs and other unrelated compounds were found to be PAMs at the M_1, M_2, M_3, and/or M_4 subtypes (Lazareno et al., 1998; Lazareno et al., 2000; Lazareno et al., 2002). More recently, compounds with higher affinities, better selectivities, and greater positive cooperativities have been identified through high-throughput screening assays with M_4 and M_1 receptors (Shirey et al., 2008; Marlo et al., 2009). Additionally,

Bridges et al. (2009) reported an M_5-selective PAM that acts by enhancing the potency of ACh, without affecting efficacy.

Despite the emphasis on the ability of PAMs to enhance agonist affinities, it has long been noted that allosteric ligands should be capable of modulating efficacy as well as potency (Ehlert, 1988), and more recent models have incorporated this potential explicitly (Hall, 2000; Ehlert, 2005). The M_4-selective PAM VU100010 has been shown to enhance both the potency and efficacy of ACh in a calcium-mobilization assay (Shirey et al., 2008). To our knowledge, amiodarone is the first compound shown to enhance receptor efficacy at the M_5 receptor, and the first muscarinic PAM to be shown to enhance efficacy without enhancing potency. Lazareno et al. (1998) introduced the term *absolute selectivity*, to describe a type of selectivity that would not depend upon the affinity of the allosteric ligand for the receptor, but rather upon the degree of cooperativity between the allosteric modulator and ACh. That is, a compound that exerts neutral cooperativity toward all but one of the receptor subtypes would be selective for that subtype irrespective of its binding affinity to the other subtypes. These authors did not restrict the term to the modulation of potency, and it is reasonable to also apply the terminology to modulation based on efficacy.

The ability of allosteric ligands to alter the rates of dissociation of orthosteric ligands to different extents has been used by several authors to compare the sites of action of allosteric ligands. If two ligands compete at a common allosteric site, but exert significantly different effects at saturating concentrations, then the effects of one ligand should be able to be reversed by the other ligand, in a strictly concentration-dependent manner. Such experiments have suggested that a large number of muscarinic allosteric

ligands (including gallamine, obidoxime, alcuronium, W-84, and strychnine and its related compounds, such as brucine) bind to a common site (Ellis and Seidenberg, 1992, Ellis and Seidenberg, 2000; Trankle and Mohr, 1997; Trankle et al., 2005). Complementary mutagenesis experiments have indicated that this common site is located within the extracellular regions of muscarinic receptors, with amino acids in the second and third extracellular loops being most important (Ellis et al., 1993; Voigtlander et al., 2003; Huang et al., 2005). Another site has been defined pharmacologically for compounds related to WIN 62,577 and for compounds related to staurosporine (Lazareno et al., 2002), although the molecular location of this site has not been investigated. The allosteric effect of amiodarone is not mediated via the well-characterized "common" site (that binds gallamine and obidoxime), but the relationship between amiodarone's binding site and the "WIN" site is not yet known.

In summary, amiodarone interacts with muscarinic receptors but is not competitive with orthosteric ligands. Rather, its ability to modulate the rate of dissociation of the orthosteric antagonist NMS and its inability to completely inhibit the binding of NMS indicate that it interacts at an allosteric site on the receptor. This interaction is novel in a number of ways. The site is different from the well-characterized one at which gallamine and obidoxime interact. Further, amiodarone's interaction leads to a selective enhancement of agonist-induced response at M_5 receptors (relative to M_1 receptors). Finally, the enhancement at the M_5 receptor is brought about by an enhancement of agonist efficacy, with no increase in potency.

Figure S1a. M_1

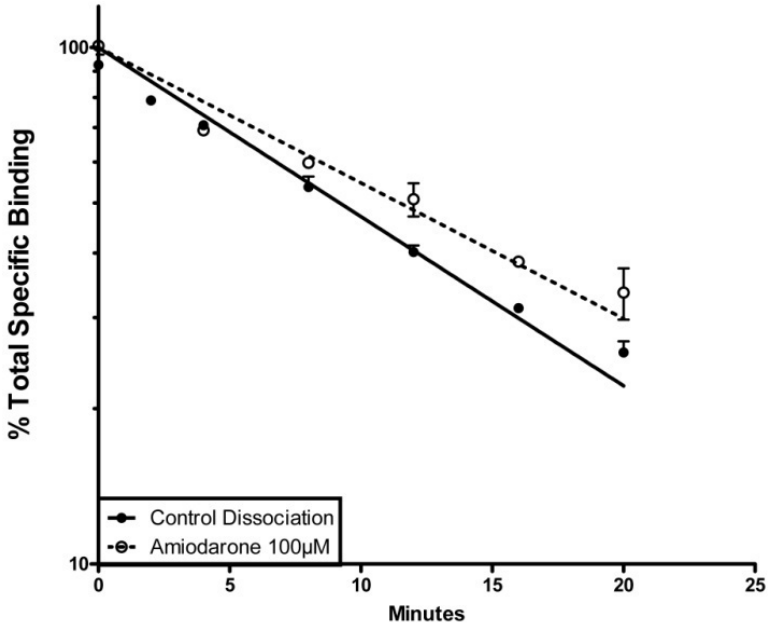


Figure S1b. M_5

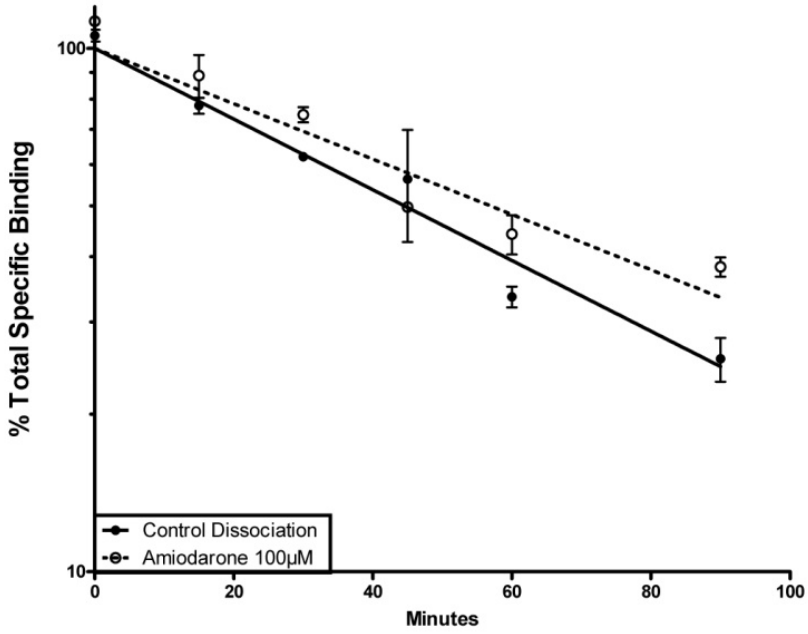


Figure 3.S1. [^{3}H]NMS dissociation is monoexponential. [^{3}H]NMS dissociates from M_1 (Panel A) and M_5 (Panel B) receptors in a manner that is consistent with a monoexponential function, in the absence (●) and presence (○) of 100 μM amiodarone. This is demonstrated as a linear fit when plotted on a logarithmic Y-axis. After establishing this, it was possible to implement the "two-point" approach to analyze the allosteric nature of amiodarone's interaction with the receptor (see *Section 2.4*). Each data point is the average of two to four experiments ± SEM.

Figure S2. M_2

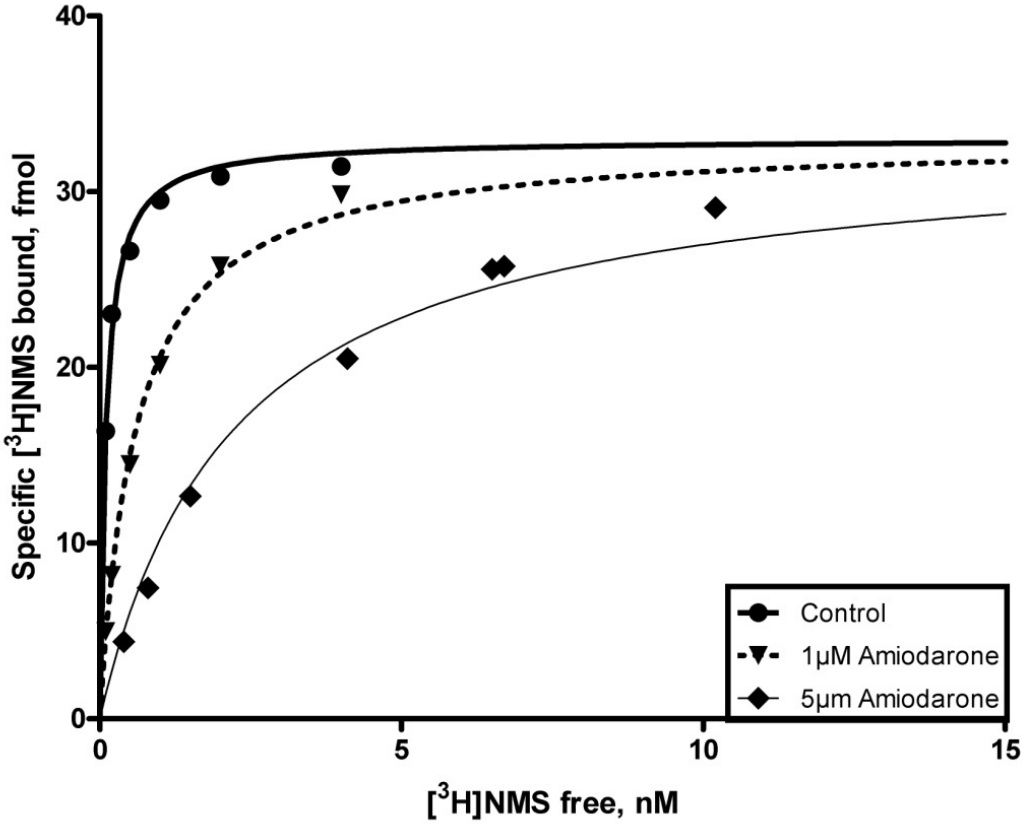
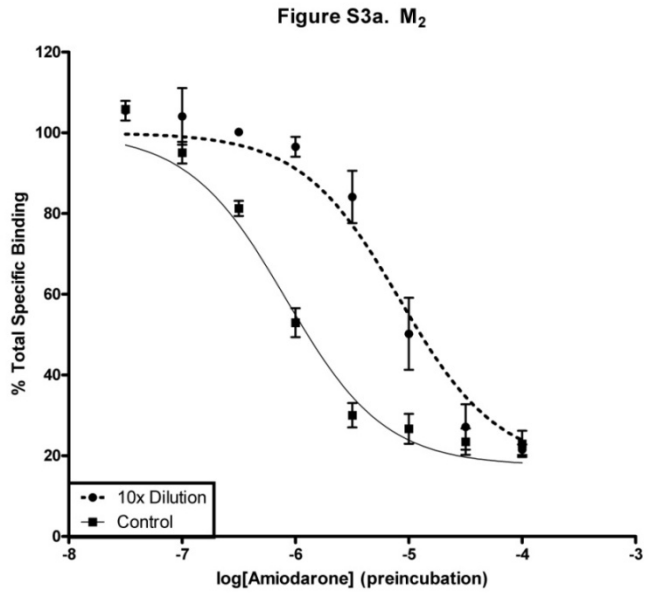
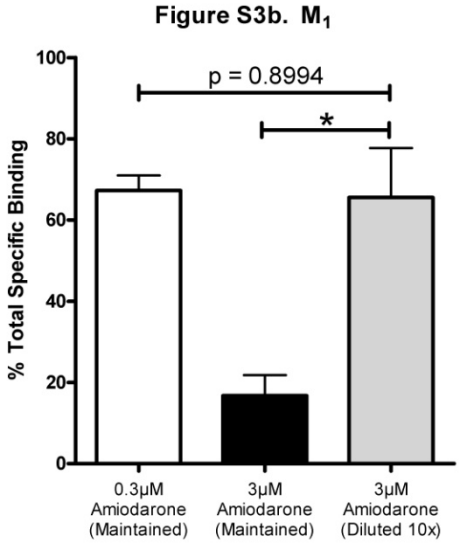


Figure 3.S2. Amiodarone does not reduce the B_{max} of [^{3}H]NMS at M_2 muscarinic receptors. The presence of 1 μM and 5 μM amiodarone progressively shifts the binding curve for [^{3}H]NMS to the right (more than 20-fold at 5 μM amiodarone) with no loss in the number of binding sites (B_{max}). The curves shown are the best fits to a common B_{max}; allowing independent values for B_{max} for the three curves did not significantly improve the fit [$F(2,25) = 2.061$, $p = 0.1484$]. Data are from two experiments, carried out in a similar manner to the equilibrium experiments in Figure 3.1.





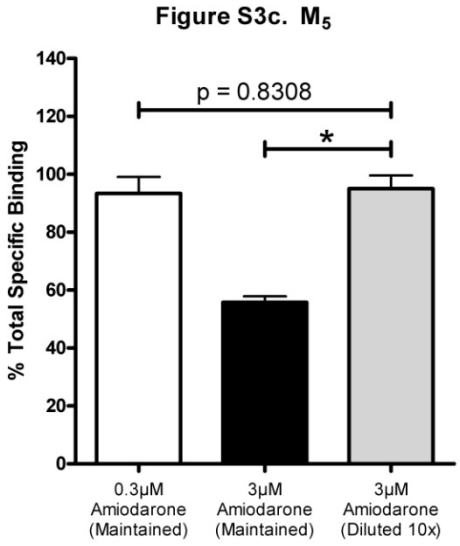
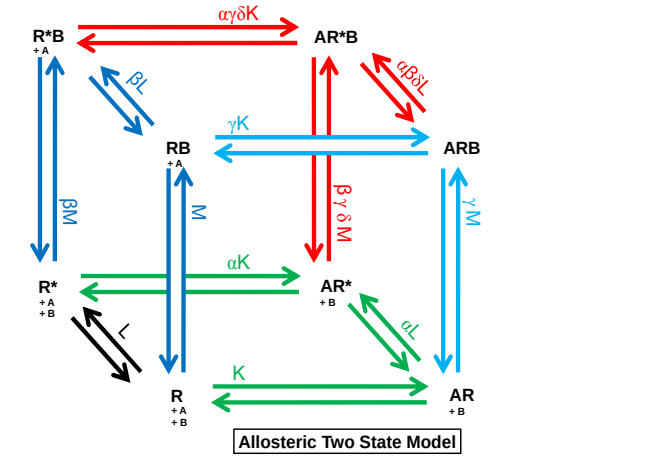


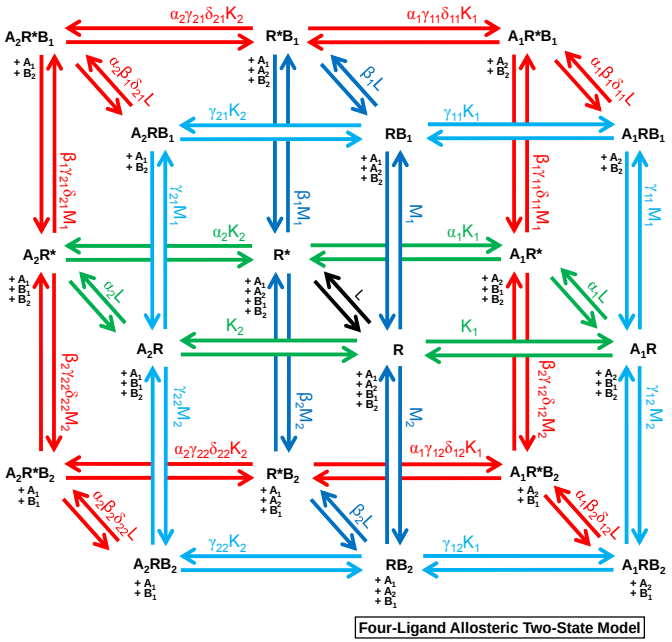
Figure 3.S3. Amiodarone binds to muscarinic receptors in a reversible manner. The inhibition of [^{3}H]NMS binding by amiodarone was reversed by simple dilution. The paradigm employed was to preincubate the membranes for an hour with amiodarone and then dilute the preparation ten-fold into either of two binding assays with [^{3}H]NMS. In one assay, the concentration of amiodarone was maintained, whereas in the other it was diluted by 10-fold. Conditions were otherwise similar to the equilibrium experiment in Figure 3.1. At the M_2 receptor (Panel A), statistical analysis of the curve-fitting found that allowing the affinities of the two curves to be independent did not give a better fit than the alternative of constraining them to be exactly 10-fold different [$F(1,44) = 0.2196$, $p = 0.6417$]. In panels B and C, M_1 and M_5 receptors were preincubated with concentrations of amiodarone that were effective at inhibiting [^{3}H]NMS binding, followed by either 10x dilution or maintenance of the indicated concentration. The binding after 10-fold dilution was not statistically different from the 10-fold lower maintained concentration of amiodarone. Each data point is the average of three experiments. *$p < 0.01$, for the comparison of diluted vs. maintained concentration

Chapter 4

Allosteric modulation of the M_3 muscarinic receptor by amiodarone and N-ethylamiodarone: application of the four-ligand allosteric two-state model



$$\text{Response}=\frac{L(1+\alpha KA+\beta MB+\alpha\beta\gamma\delta KAMB)}{1+KA+MB+\gamma KAMB+L(1+\alpha KA+\beta MB+\alpha\beta\gamma\delta KAMB)}$$



$$\text{Response}=\frac{\begin{array}{c}L(1+\alpha_1K_1A_1+\alpha_2K_2A_2+\beta_1M_1B_1+\beta_2M_2B_2\\+\alpha_1\beta_1\gamma_{11}\delta_{11}K_1A_1M_1B_1+\alpha_2\beta_1\gamma_{21}\delta_{21}K_2A_2M_1B_1\\+\alpha_1\beta_2\gamma_{12}\delta_{12}K_1A_1M_2B_2+\alpha_2\beta_2\gamma_{22}\delta_{22}K_2A_2M_2B_2)\end{array}}{\begin{array}{c}1+K_1A_1+K_2A_2+M_1B_1+M_2B_2\\+\gamma_{11}K_1A_1M_1B_1+\gamma_{12}K_1A_1M_2B_2+\gamma_{21}K_2A_2M_1B_1+\gamma_{22}K_2A_2M_2B_2\\+L(1+\alpha_1K_1A_1+\alpha_2K_2A_2+\beta_1M_1B_1+\beta_2M_2B_2\\+\alpha_1\beta_1\gamma_{11}\delta_{11}K_1A_1M_1B_1+\alpha_1\beta_2\gamma_{12}\delta_{12}K_1A_1M_2B_2\\+\alpha_2\beta_1\gamma_{21}\delta_{21}K_2A_2M_1B_1+\alpha_2\beta_2\gamma_{22}\delta_{22}K_2A_2M_2B_2)\end{array}}$$

Figure 4.1. The allosteric two-state model and the four-ligand allosteric two-state model. The allosteric two-state model (ATSM) is presented in the upper-left panel (Hall, 2000). The associated equation presents the response equation Hall developed from this model, color-coded in order to better illustrate its relationship to the model. The four ligand allosteric two-state model (4L-ATSM), in the lower-left panel, is an extension of the ATSM that can accommodate two orthosteric ligands (A_1 and A_2), and two allosteric ligands (B_1 and B_2), such that there is competition between two ligands at each binding site. The associated equation reflects the response equation for this new model, similarly color-coded to articulate its relationship to the original ATSM response equation. The parameters used in the new model are defined in Table 2.1.

Stahl and Ellis (2010) presented the characterization of the allosteric effects of amiodarone on muscarinic receptors and established that it interacts with a novel site. An enhancement of the M_5 response was observed for a number of full and partial muscarinic agonists, including ACh. When amiodarone was included in ACh concentration-response curves, there was an increase in the level of maximum response elicited from M_5 receptors. The allosteric nature of this interaction was supported by binding studies that demonstrated amiodarone did not interact with the orthosteric ACh binding site.

This chapter presents a more complete representation of the allosteric effects of amiodarone by focusing on the M_3 receptor. To begin with, amiodarone inhibits the binding of N-methylscopolamine (NMS) in an allosteric manner at the M_3 subtype. Despite this negative cooperativity toward NMS, amiodarone is referred to as a positive allosteric modulator (PAM) throughout this chapter, based on its ability to enhance functional responses stimulated by the endogenous agonist, ACh. Two different responses mediated by the M_3 receptor were used to investigate the PAM activity of amiodarone. Although it initially appeared that amiodarone only enhanced ACh's action in one of the responses, reduction of the magnitude of receptor reserve (by receptor alkylation) reveals similar PAM activity in both measures of response. Finally, by applying simulations generated from the newly developed four-ligand allosteric two-state model (4L-ATSM; Figure 4.1), it can be concluded that amiodarone and its quaternary analog N-ethylamiodarone (NEA; Figure 4.2) act at a common allosteric site on the extracellular domain of the receptor.

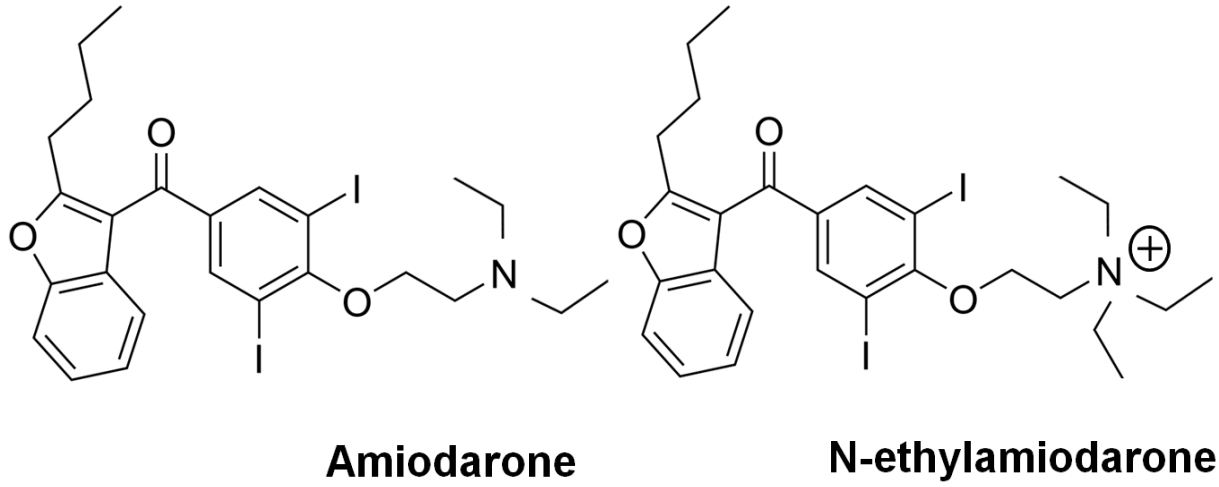


Figure 4.2. Molecular structures of the two allosteric ligands studied in this chapter.

4.2 Results

4.2.1 Amiodarone inhibits [^{3}H]NMS binding at the M_3 muscarinic receptor

The previous chapter indicated that, at the M_1, M_2, and M_5 subtypes, amiodarone produced incomplete inhibition of [^{3}H]NMS binding. In this chapter, the inhibition of the binding of 1 nM [^{3}H]NMS was examined at the M_3 subtype. As presented in Figure 4.3, amiodarone inhibits [^{3}H]NMS binding with a logIC_{50} value of -5.3 ± 0.2. In agreement with the previously reported findings, amiodarone inhibits [^{3}H]NMS binding in an incomplete manner, the lower asymptote for inhibition being significantly different from zero. That is, amiodarone inhibits [^{3}H]NMS binding to only about 65% of specific binding. Under these conditions, it is possible to determine the affinity of amiodarone (logK_A) and cooperativity between amiodarone and NMS (α) using the TCM. The best-fit values from the model are log$K_A = -5.8$ and $\alpha = 0.25$.

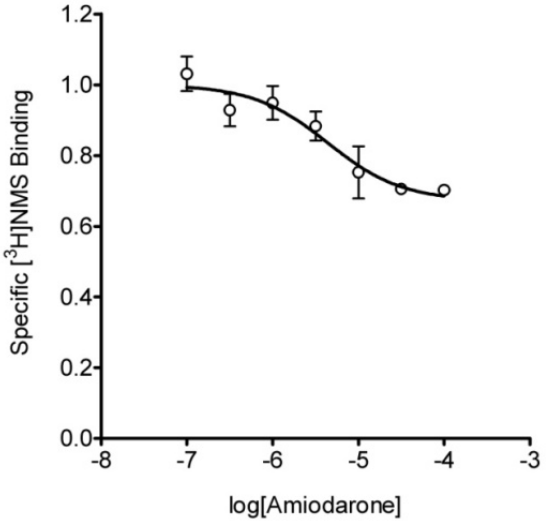


Figure 4.3. [^{3}H]NMS equilibrium binding is incompletely inhibited by amiodarone. Amiodarone inhibited [^{3}H]NMS binding at the M_3 receptor. The bottom plateau of the binding inhibition curve is significantly different from zero ($p < 0.0001$). Each point is the average of three experiments expressed as mean ± SEM.

4.2.2 The level of maximal AA release, for ACh and pilocarpine, is enhanced in the presence of amiodarone at M_3 receptors

In Figure 4.4A, 10 μM amiodarone elevates the maximal release of AA elicited via M_3 receptor activation. The maximal response to ACh is elevated by 48 ± 7.5% and pilocarpine's maximal response is elevated by 105 ± 5.5% (the maximal response for pilocarpine was 14 ± 0.8% of the maximum for ACh). In Figure 4.4B, 30 μM amiodarone caused elevation of the maximal response of ACh, at M_3, by 70% over acetylcholine alone; this increase was highly significant (see the legend of Figure 4.4). The ACh potency in the presence of amiodarone ($\log EC_{50}$ = ‑6.9 ± 0.05) was shifted leftward only slightly with respect to the control curve ($\log EC_{50}$ = ‑6.8 ± 0.07); this shift did not achieve significance.

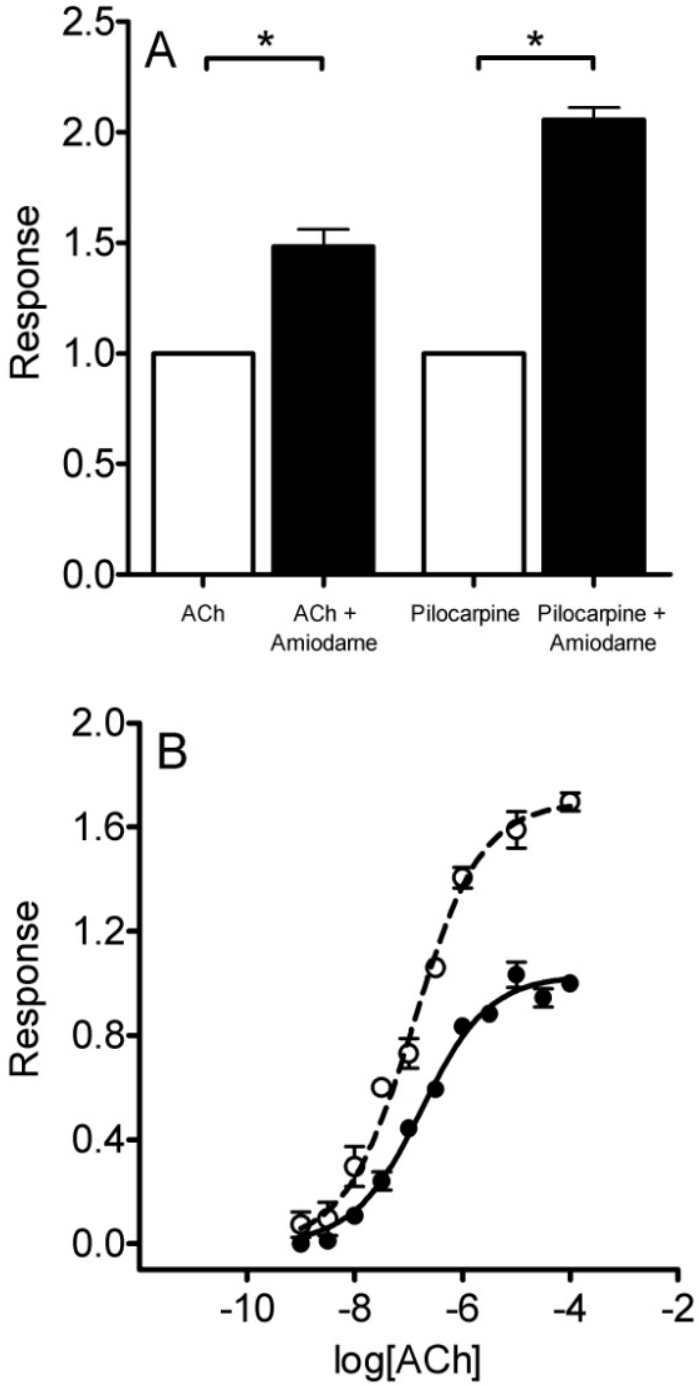


Figure 4.4. Amiodarone enhances agonist stimulated [^{3}H]AA release at the M_3 receptor. In panel A, 10 μM amiodarone displays allosteric effects which cause the elevation of the response stimulated by 100 μM ACh and 1 mM pilocarpine. The effect of amiodarone on the M_3 concentration-response curve of ACh is presented in panel B. The response stimulated by ACh is presented in the absence (closed circles) and presence (open circles) of 30 μM amiodarone. Release of [^{3}H]AA stimulated by ACh was elevated in the presence of amiodarone ($p < 0.0001$). ACh potency was slightly enhanced, but this enhancement did not achieve significance. Each point is the average of three experiments expressed as mean ± SEM. *$p < 0.05$

4.2.3 Amiodarone enhances maximal IP metabolism stimulated by pilocarpine, but not ACh, at the M_3 receptor

Amiodarone also exhibited PAM activity on agonist stimulated IP metabolism. In the case of pilocarpine, amiodarone caused a significant elevation of the maximal level of IP metabolism (Figure 4.5A). The $\log EC_{50}$ value for pilocarpine was also shifted leftward from -5.9 ± 0.07 to -6.2 ± 0.05 in the presence of amiodarone. Pilocarpine stimulated IP metabolism to 65 ± 3.3% of the maximal response for ACh. The presence of 30 μM amiodarone did not enhance the maximal response elicited by ACh, but there was about a 2-fold leftward shift in ACh potency, from $\log IC_{50}$ values of -7.4 ± 0.04 to -7.8 ± 0.05 (Figure 4.5B). These findings demonstrate that amiodarone affected IP metabolism and AA release in a qualitatively similar manner; in both cases, amiodarone acts as a PAM. However, these studies do raise the question of why the maximal stimulation of IP metabolism by ACh was not enhanced by amiodarone.

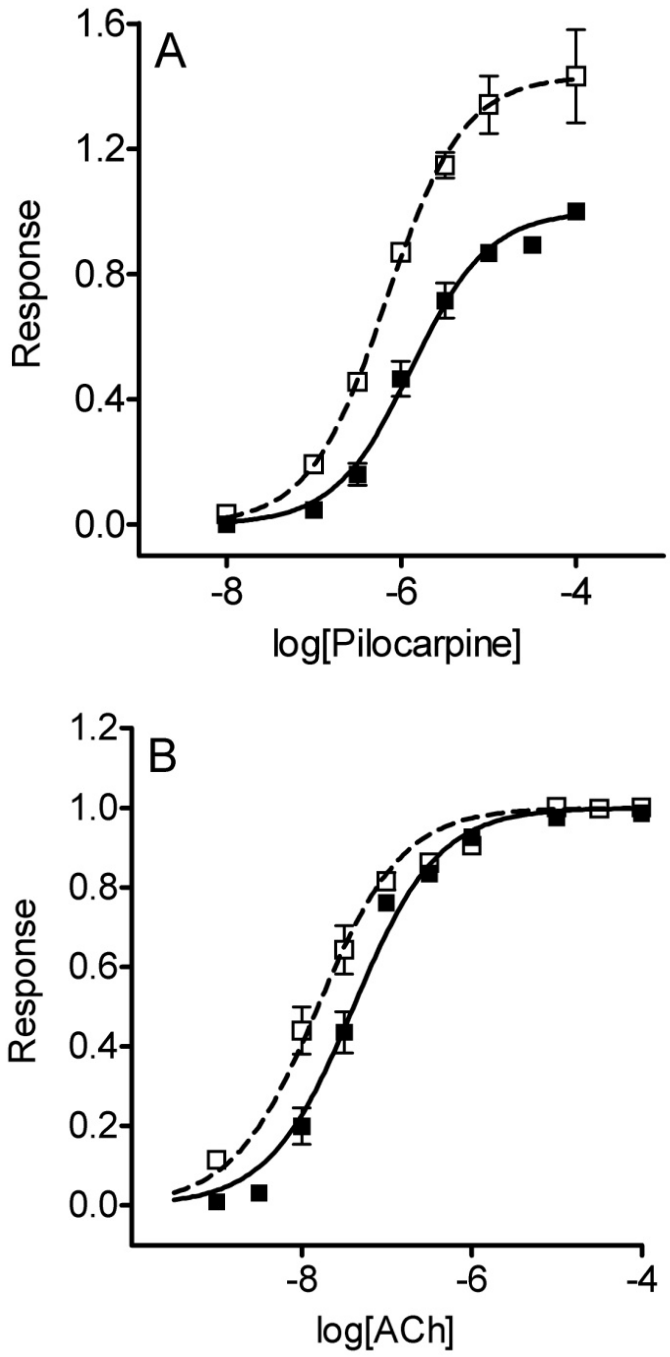


Figure 4.5. Amiodarone enhances the maximal stimulation of [^{3}H]IP metabolism induced by pilocarpine at the M_3 receptor. IP metabolism stimulated by pilocarpine (panel A) and ACh (panel B) is presented in the absence (closed squares) and presence (open squares) of 30 μM amiodarone. In the presence of amiodarone, the maximal response stimulated by pilocarpine was elevated but maximal stimulation by ACh was not enhanced. Amiodarone did cause an increase in potency of both ACh and pilocarpine (see *Section 4.2.3*). Each point is the average of three experiments expressed as mean ± SEM.

4.2.4 IP response is subject to a larger receptor reserve than AA response

The ability of amiodarone to enhance response in only one of the two response assays may be the result of different receptor reserves. This was investigated by determining the maximal response obtained in IP metabolism and AA release, after pretreatment with increasing concentrations of the irreversible antagonist phenoxybenzamine (POB). Treatment with POB has been shown to cause a concentration-dependent loss of muscarinic binding sites (Eglen and Harris, 1993; Leff et al., 1993). In Figure 4.6, maximal response (at saturating agonist concentration) is presented as a function of the fraction of [^{3}H]NMS binding sites remaining after POB pretreatment. Based on [eq. 5], the best-fit values for *r* were determined in this figure (Table 4.1); it is clear that ACh benefits from a much larger receptor reserve in the IP response than it does in the AA response. Of the four curves shown in Figure 4.6, only one, the ACh/IP response, can be said to have clearly reached a ceiling of maximal effect. Accordingly, a large proportion of receptors must be irreversibly alkylated before maximal response is measurably reduced. On the other hand, only the pilocarpine/AA response is directly proportional to occupancy (i.e. r = 1). Also shown in Table 4.1 are the EC_{50} values for ACh and pilocarpine at the two responses. In each case, the K_A values are calculated (as EC_{50} / r). Different K_A values could indicate the presence of different receptor conformations. Interestingly, ACh does display a 4-fold higher affinity for the receptor in mediating the AA response, relative to its affinity in mediating the IP response. One advantage of this approach is that it is not necessary to make assumptions as to whether a given agonist has the same K_A toward different responses

(if different responses are due to different conformations of the receptor, there is no reason that K_A must be a shared value; see Hall and Langmead, 2010).

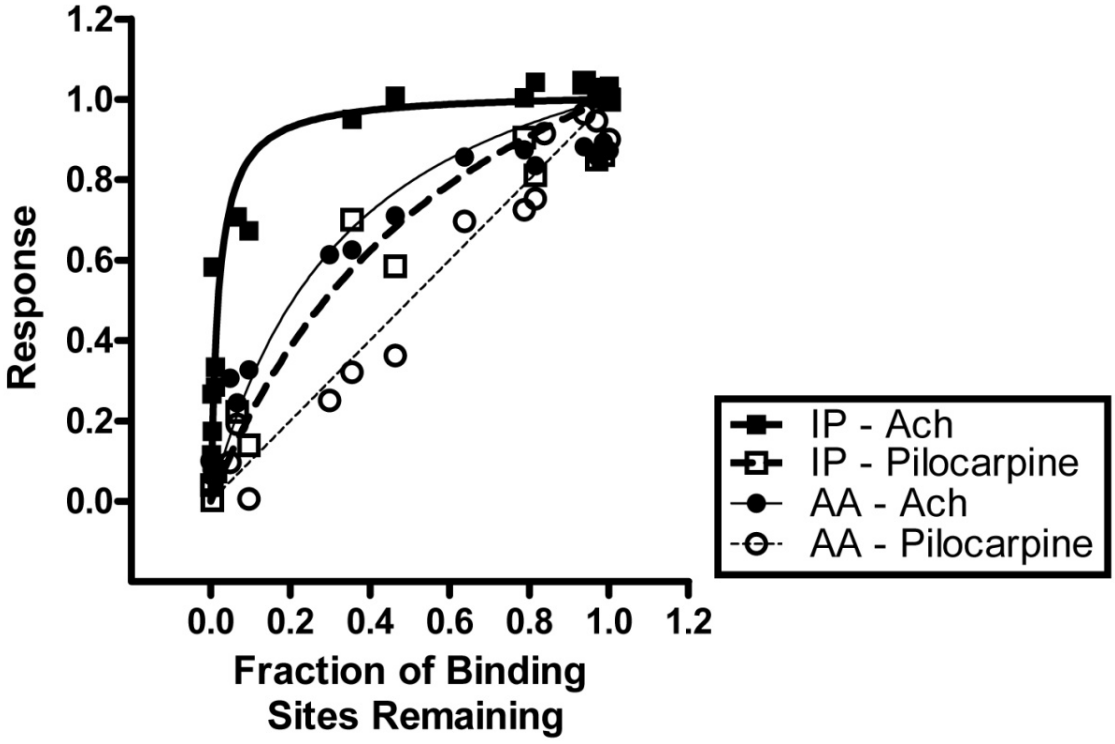


Figure 4.6. AA release and IP metabolism exhibit different degrees of receptor reserve. Stimulation of both responses was measured with saturating concentration of a full or partial agonist after pretreatment with POB. These measures of response, after POB treatment, are expressed as a function of the fraction of receptors remaining (measured by binding of 1 nM [^{3}H]NMS to intact cells). For IP metabolism, ACh exhibited a large receptor reserve, whereas the partial agonist pilocarpine experienced a much smaller degree of reserve. For AA release, ACh exhibited a much smaller degree of receptor reserve, similar to that of pilocarpine in IP metabolism. Pilocarpine did not exhibit any receptor reserve in stimulating AA release. Data points are the individual points from three different experiments. The best-fit curves are generated using [eq. 5] and the *r* values are presented in Table 4.1.

Table 4.1. Parameter values obtained from the quantification of receptor reserve in Figure 4.6.

Response	Agonist	*r* Value	logEC_{50}[a]	Calculated logK_A Value[b]
AA release	ACh	0.3 ± 0.04*	-6.8 ± 0.07	-6.3
	Pilocarpine	1.1 ± 0.14	-5.7 ± 0.04	-5.7
IP metabolism	ACh	0.02 ± 0.004*	-7.4 ± 0.04	-5.7
	Pilocarpine	0.4 ± 0.08*	-5.9 ± 0.07	-5.5

[a] EC_{50} values are from the data presented in Figures 4.4B, 4.5A, 4.5B, and 4.8

[b] K_A values calculated as EC_{50} / *r*

* value significantly different from 1; $p < 0.05$

4.2.5 Following POB pretreatment that significantly reduces IP and AA response, amiodarone can potentiate the maximal stimulation of both responses by ACh

Based on initial studies, 1 μM POB was selected as an effective concentration of pretreatment for investigating the influence of receptor reserve on the response studies. After pretreatment with POB, application of 30 μM amiodarone along with ACh caused a significant elevation of the maximal level of IP metabolism (and AA release, as would be expected), compared to that of ACh alone (Figure 4.7A). In light of these findings, a more complete picture of the effects of amiodarone on ACh stimulated IP metabolism was pursued. Cells were pretreated with POB followed by generation of ACh concentration-response curves in the presence and absence of amiodarone. In Figure 4.7B, POB pretreatment caused a rightward shift in ACh potency to $\log EC_{50}$ -6.0 ± 0.06, as would be expected with a marked reduction of the receptor reserve. Amiodarone caused a leftward shift of potency to -6.3 ± 0.05. Amiodarone not only produced enhancement in ACh potency but also elevated the maximal response stimulated by ACh from 60 to 76% of the control maximal response (a relative increase of 25%).

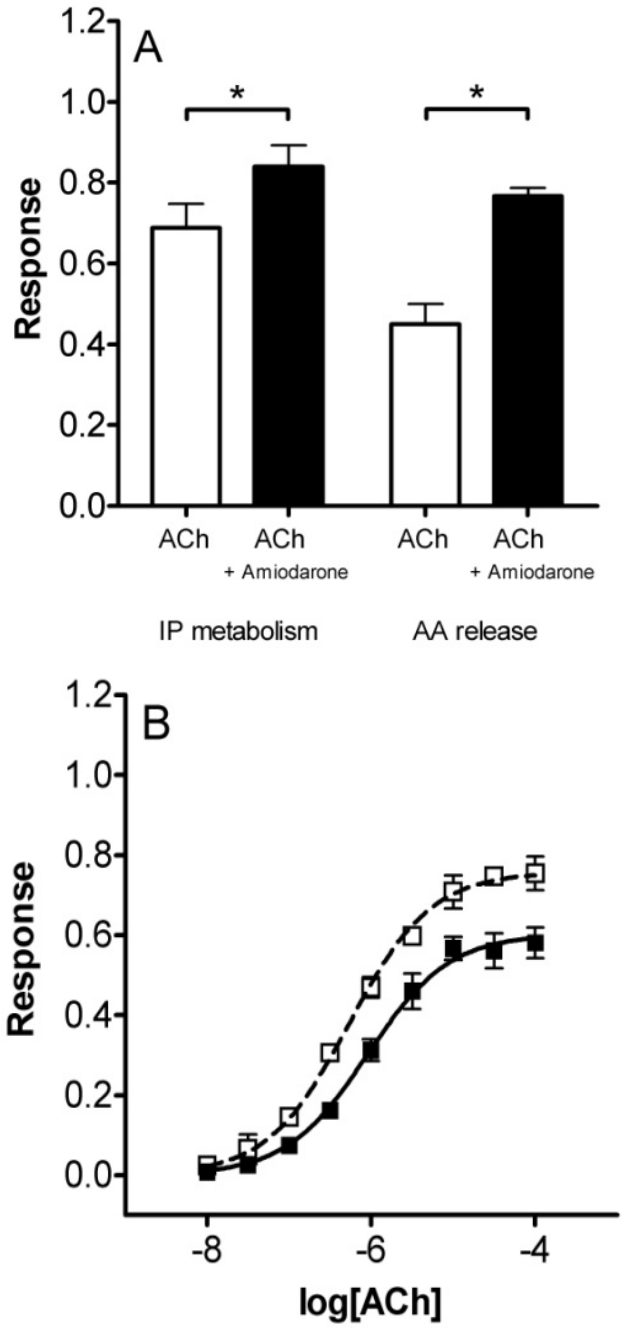


Figure 4.7. Following POB pretreatment, amiodarone causes elevation of the maximal IP metabolism and AA release stimulated by the M_3 receptor. 1 μM POB pretreatment was found to be effective at inhibiting the ACh maximal response. In panel A, upon POB pretreatment, ACh maximal response was inhibited by 32% for IP metabolism and 55% for AA release. After pretreatment, amiodarone caused significant elevation of both responses (*$p < 0.05$). In panel B, ACh concentration-response curves demonstrate the inhibitory effect of POB pretreatment on ACh mediated IP metabolism (solid squares) and the significant potentiation caused by amiodarone on both ACh potency and maximal response (open squares; $p < 0.01$). Each point is the average of three experiments expressed as mean ± SEM.

4.2.6 NEA reverses amiodarone's potentiation of maximal response at the M_3 receptor

It was of interest to investigate the interactions between amiodarone and an agonist in the presence of an additional orthosteric or allosteric agent, and to compare these experiments with the predictions of the 4L-ATSM. Stimulation of the AA response by pilocarpine was the preferred choice in these studies because the analysis of receptor reserve had demonstrated that it was in agreement with the occupancy model (Figure 4.6). Additionally, amiodarone caused a larger enhancement of maximal AA release when pilocarpine was used as the agonist (Figure 4.4A). In Figure 4.8 (both panels), amiodarone enhanced the maximal degree of AA release that pilocarpine was able to induce via the M_3 receptor. The $\log EC_{50}$ of pilocarpine (-5.7 ± 0.04) was not significantly enhanced by the presence of amiodarone. NMS is a competitive antagonist and interacts with the orthosteric binding site on the receptor. As would be expected, NMS caused a rightward shift in the $\log EC_{50}$ of pilocarpine, to -4.2 ± 0.03. NMS also produced a rightward shift in the pilocarpine response curve in the presence of amiodarone ($\log EC_{50}$ -4.3 ± 0.04), but the elevation of the maximal response caused by amiodarone was not affected. The quaternary analog of amiodarone, NEA, was also investigated using this paradigm. NEA (Figure 4.8B) caused a parallel rightward shift in the pilocarpine curve, of similar magnitude to that caused by NMS ($\log EC_{50} = -4.0 \pm 0.04$). However, unlike NMS, NEA markedly attenuated amiodarone's enhancement of the pilocarpine maximal response; NEA shifted the $\log EC_{50}$ for this curve to -4.2 ± 0.03.

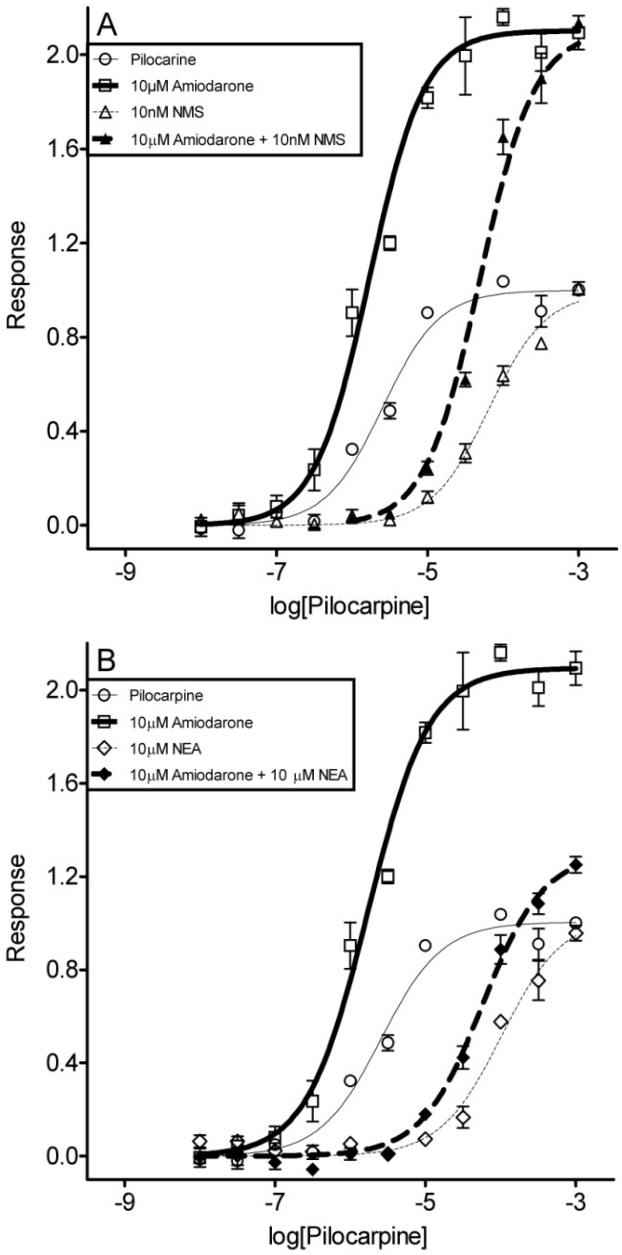


Figure 4.8. NEA and NMS exhibit qualitative differences in their modulation of amiodarone's effect. In panels A and B, the [^{3}H]AA release mediated by pilocarpine at M_3 is presented in the absence (open circles) and presence (open squares) of 10 μM amiodarone. Amiodarone caused an increase in the maximal response without a significant change in pilocarpine potency. In panel A, the concentration-response curve of pilocarpine was rightward shifted by the competitive antagonist NMS (open triangles). In the presence of amiodarone, NMS caused a similar rightward shift in the response curve but did not reduce the enhancement of maximal response caused by amiodarone (closed triangles). In panel B, 10 μM NEA caused a rightward shift in the concentration-response curve of pilocarpine (open diamonds). The pilocarpine response curve in the presence of amiodarone was also shifted rightward by NEA and, moreover, the enhancement of maximal response caused by amiodarone was markedly attenuated by NEA (closed diamonds). Each point is the average of three experiments expressed as mean ± SEM.

4.2.7 Simulations of the four-ligand allosteric two-state model

The 4L-ATSM (Figure 4.1; see *Section 4.4.1* for derivation) is a new expansion of the ATSM that describes the functional consequences of two ligands competing at the orthosteric site and two different ligands competing at the allosteric site. In the 4L-ATSM, the central receptor transition (R⇌R*) represents the isomerization from the inactive to the active state. The peripheral receptor states indicate multiple bound ligands that can modify that isomerization. The associated equation in Figure 4.1 is the functional consequence of the model, color-coded to illustrate the relatedness of the equation to the model and also to Hall's original model. Simulations were generated from this equation in order to illustrate the predictions of the model when applied to the experimental paradigms used in Figure 4.8. These two paradigms were: the presence of one allosteric and two orthosteric ligands; or, the presence of one orthosteric and two allosteric ligands. The parameter values in Table 4.2 were selected so that the theoretical ligands would be comparable to pilocarpine (A_1), NMS (A_2), amiodarone (B_1), and NEA (B_2).

Competition between an orthosteric agonist and a neutral orthosteric antagonist causes a rightward shift in the agonist concentration-response curve; this characteristic is evident in the simulation shown in Figure 4.9A. The orthosteric antagonist also produces the expected rightward shift in the presence of a positive allosteric modulator (PAM) that elevates maximal response, but the orthosteric antagonist does not reverse the elevation of the upper asymptote caused by the PAM. Conversely, competition between two ligands at a common allosteric site will produce response curves of a much different nature (Figure 4.9B). The presence of the negative allosteric modulator

(NAM) causes a rightward shift from the control agonist response curve. When the same inhibitory concentration is applied to agonist response, in the presence of the PAM, it is clear that this interaction differs from the case of the orthosteric antagonist. In this PAM + NAM curve, the elevation of the upper asymptote is attenuated by the presence of the NAM, due to the competition for occupancy of the common allosteric site. The striking similarities between these simulations and the experimental data in Figure 4.8 reinforce the expectation (based on their common structure) that amiodarone and NEA compete at a common allosteric site.

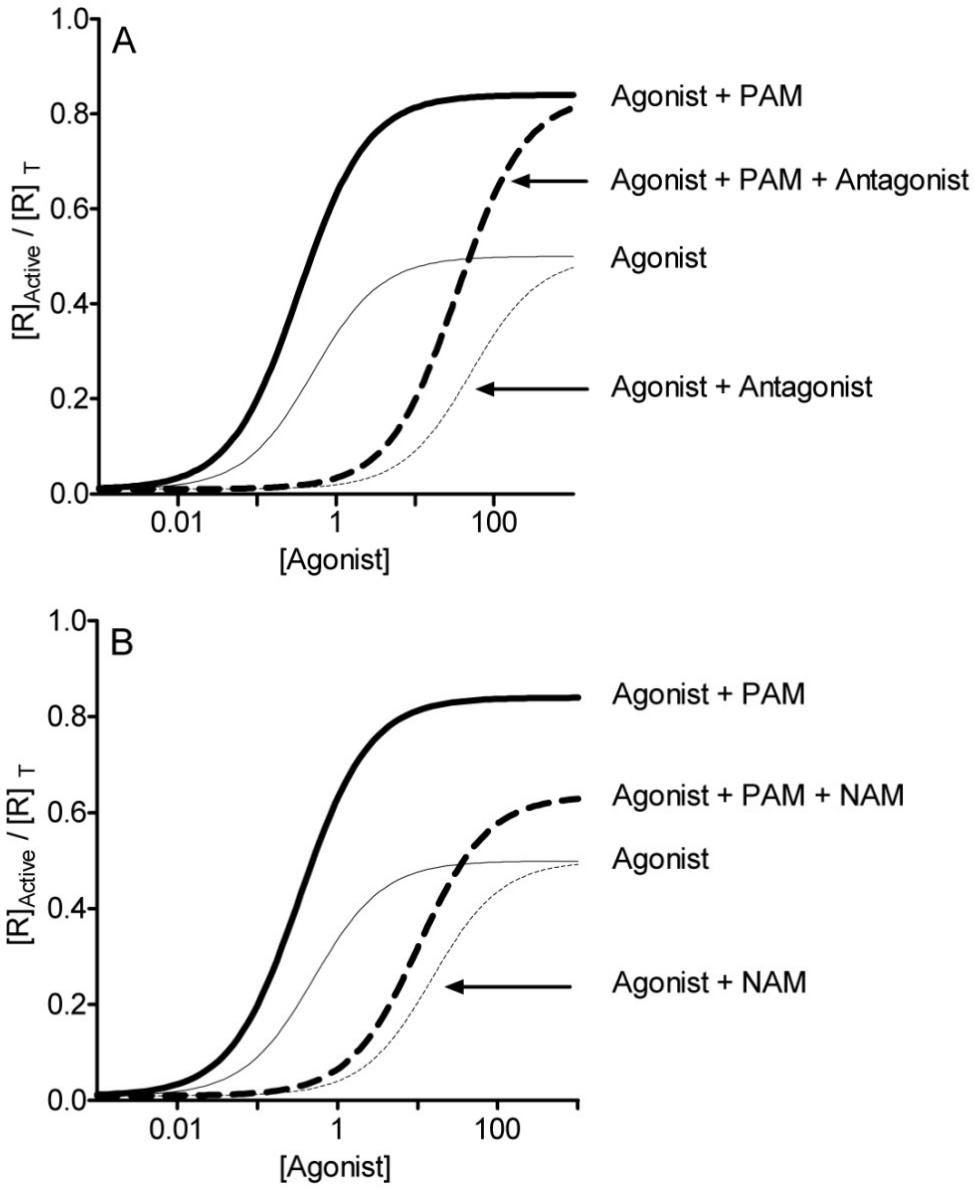


Figure 4.9. Simulations of the 4L-ATSM illustrate the effects of competition at either the orthosteric or allosteric site. In panels A and B, the effects of an agonist alone are represented by the solid thin line (——), whereas the effects of the agonist in the presence of a PAM are represented by the solid thick line (▬▬). In panel A, a competitive antagonist causes a rightward shift in the agonist response curve in the presence (▪▪▪▪▪) and absence (.....) of the PAM. The competitive antagonist is not able to reverse the enhancement of the upper asymptote caused by the PAM. In panel B, a NAM that is neutral on efficacy causes a rightward shift in the agonist response curve in the absence (.....) and presence (▪▪▪▪▪) of a PAM. Notably, the enhancement of the upper asymptote caused by the PAM is reversed by the NAM. Table 4.2 indicates the parameter values which were applied to the 4L-ATSM equation in Figure 4.1 for these simulations.

Table 4.2. Parameter values used to develop the simulations presented in Figure 4.9.

Parameter Name	Simulation Values
K_1	1
K_2	1
L	0.01
M_1	1
M_2	1
α_1	100
α_2	1
β_1	1
β_2	1
γ_{11}	0.3
γ_{12}	0.03
γ_{21}	1
γ_{22}	–
δ_{11}	10
δ_{12}	1
δ_{21}	1
δ_{22}	–

4.3 Discussion

The ability of amiodarone to inhibit the binding of [^{3}H]NMS to M_1, M_2, and M_5 muscarinic receptors has been previously reported (Stahl and Ellis, 2010). The work presented in this chapter extends the study of amiodarone's effects on binding to include the M_3 subtype (Figure 4.3). In agreement with the proposed allosteric mechanism of action, amiodarone does not completely inhibit binding at the M_3 subtype. Incomplete binding inhibition is convincing evidence that a ligand does not compete for the same site as the radioligand (Christopoulos and Kenakin, 2002). In the application of allosteric theory, the focus has usually been on binding cooperativity, a point embodied in the TCM, in which the binding cooperativity is a shared modulatory value that the two ligands possess when they are bound to a common receptor. However, it is recognized that allosteric ligands are capable of modulating efficacy (Ehlert, 1988) and a number of groups have observed allosteric modulation of agonist efficacy at GPCRs (Urwyler et al., 2003; Sharma et al., 2008; Bridges et al., 2009). Our previous studies found that amiodarone was able to allosterically enhance agonist-stimulated response at the M_5 receptor, without any potency enhancement. The studies presented in this chapter demonstrate that amiodarone potentiated AA release mediated by the M_3 receptor (Figure 4.4). This enhancement in maximal response was accompanied by a trend towards a leftward shift in ACh potency. These findings demonstrate that amiodarone affects M_3 mediated response in a manner similar to M_5.

This chapter shows that amiodarone causes elevation of maximal ACh mediated AA release but not maximal ACh mediated IP metabolism (Figure 4.4B and 4.5B). One explanation for differential potentiation is that IP metabolism is subject to a large

receptor reserve and the maximal response is therefore not able to be elevated, whereas stimulation of AA release is not subject to such a significant receptor reserve. The ability of amiodarone to enhance both responses elicited by the partial agonist pilocarpine is in agreement with this concept. The finding of markedly different reserves suggests in turn that the two responses are independent, not sequential. Furthermore, ACh displays different K_A values for the responses (Table 4.1); this implies that different conformations of the receptor are responsible for the two responses. In fact, there is a large body of evidence in the literature demonstrating the independence of these responses. For example, Burch et al. (1986) found that both responses were linked to α_1-adrenergic receptors via G proteins, but that different agents could inhibit one response without affecting the other. Conklin et al. (1988) reported that acute treatment of A9L cells with a PKC activator, phorbol 12-myristate 13-acetate, caused the activation of IP metabolism by M_3 receptors to be inhibited, whereas the muscarinic AA response was enhanced. Perez et al. (1996) identified a mutation in α_{1B}-adrenergic receptors that induced constitutive activity in the IP response, while the AA response was unaffected. Kurrasch-Orbaugh et al. (2003a) found that different $5HT_{2A}$ receptor agonists possessed opposite functional selectivity for the two responses in NIH-3T3 cells; indeed, these authors also found different magnitudes of receptor reserve for the two responses. Finally, Berg et al. (1998) found that $5HT_{2C}$ agonists possessed different affinities for the active states that lead to IP and AA responses in CHO cells. These authors suggested that the different affinities are the cause of the phenomenon known as agonist-directed trafficking of receptor stimulus.

It is worth noting that the use of screening assays that exhibit large receptor reserves may preclude the discovery of PAMs with properties similar to those of amiodarone. That is, the ability of amiodarone to enhance the maximal IP response elicited by ACh was not apparent until the receptor reserve was eliminated. This may be one reason why allosteric modulators which elevate maximal response without altering potency to a significant extent are so rarely discovered. Additionally, the sensitivity of this type of PAM to the degree of receptor reserve may present therapeutic advantages in some cases. For example, if a pathological condition was caused by a loss of receptors in a particular tissue that resulted in a significant reduction in the receptor reserve, such a PAM could enhance response in the affected tissue, while tissues with intact receptor reserves remain unaffected.

The remaining question is why amiodarone is so effective at enhancing the maximal effect of ACh in the AA response, even though there is an indication of some receptor reserve. The likely answer is simply that the degree of reserve is small. Comparison of the ACh/IP curve to the ACh/AA curve in Figure 4.6 illustrates that the IP response clearly reaches a ceiling as more and more receptors are left intact; the AA response possess no such ceiling effect. However, there is a further possibility. Studies have indicated that GPCRs are capable of differentially activating multiple G proteins (e.g., Akam et al., 2001) and that receptor-mediated AA release is mediated by multiple parallel pathways that are initiated by distinct G proteins (Kurrasch-Orbaugh et al., 2003b; Hecquet et al., 2006). It is possible that there is a larger receptor reserve associated with one of these pathways, and that amiodarone acts

predominantly on the pathway with the smaller degree of reserve. Further studies will be required to address this possibility.

As described in *Section 4.1*, the ATSM is a mechanistic model that is useful for understanding response modulation in the presence of an allosteric ligand. Two of the parameters of the model, binding cooperativity (γ) and activation cooperativity (δ), represent the forms of modulation an allosteric ligand may induce on agonist response curves. That is, positive values of either parameter will cause an enhancement of agonist potency and positive activation cooperativity may additionally cause an enhancement of maximal response, if there is not a system dependent limit to response. Studies of the effects of amiodarone on agonist stimulated [^{3}H]AA release at the M_3 receptor (Figure 4.8) were compared to simulations based on the response equation of the 4L-ATSM (Figure 4.9). The simulations demonstrate that the 4L-ATSM is capable of elegantly replicating the ability of amiodarone to enhance maximal response without enhancing agonist potency; this is accomplished by stipulating a *positively* cooperative δ and *negatively* cooperative γ that counterbalance each other's effect on potency (see Table 4.2). Of course, in the case of a single orthosteric and allosteric ligand, the 4L-ATSM reduces to the ATSM. Hall (2000) used the ATSM to simulate the opposite case, where efficacy is reduced due to a negative δ, but potency is unchanged due to a positive γ, to replicate results obtained by Litschig et al. (1999) at the metabotropic glutamate receptor mGluR1. In a similar set of findings, Price et al. (2005) demonstrated an allosteric inhibition of maximal response combined with an increase in agonist potency.

The most striking effect of amiodarone is its ability to enhance the maximal responses elicited by acetylcholine and pilocarpine. The quaternary analog NEA lacks this property entirely. Sharma et al. (2008) reported a similar switch from positive to negative allosteric modulation caused by differential methylation of adjacent positions in a phenyl ring in a series of mGluR5 ligands. They termed this a “molecular switch” in the compound and noted that allosteric modulators can be especially sensitive to dramatic shifts in pharmacological properties as a result of slight changes in structure. Indeed, NEA simply shifts the agonist concentration-response curves to lower apparent potency, in a manner that superficially resembles the effect produced by the orthosteric antagonist, NMS (Figure 4.8). Based on their close chemical structures, NEA would be expected to act at the same site at which amiodarone acts. The ATSM is well suited to describing response cooperativity between one allosteric ligand and one orthosteric ligand, as we have emphasized above. In order *to quantitatively describe and compare* the behavior expected of an antagonist like NMS, which interacts competitively at the orthosteric site, with an allosteric antagonist that interacts competitively with amiodarone at its allosteric site, a more expansive model was needed. Fortunately, as noted in *Section 4.1*, the ATSM shares with the CTCM the feature that it can readily be generalized. We have expanded the cubic ATSM to a tetra-cubic model that accommodates two orthosteric ligands and two allosteric ligands simultaneously; we call this four-ligand version of the model the 4L-ATSM. The two models and their equations of response are presented in a color-coded manner in Figure 4.1, to permit visual appreciation of the relationships between the models and also to articulate the relationship between the models and their respective equations. Simulations based on

the 4L-ATSM used parameter values consistent with an orthosteric agonist (A_1), an orthosteric antagonist (A_2), an allosteric ligand that promotes positive activation cooperativity but negative binding cooperativity with A_1 (B_1), and an allosteric ligand that promotes only negative binding cooperativity with A_1 (B_2). The curves resulting from these simulations are consistent with the experimental data and reinforce the expectation that amiodarone and NEA interact with a common allosteric site. Furthermore, the quaternary nature of NEA indicates that this site lies on the extracellular portion of the receptor.

In conclusion, amiodarone interacts with a novel allosteric site on muscarinic receptors. This chapter is focused on characterizing the ability of amiodarone to act as a PAM on responses elicited by activation of the M_3 receptor. This PAM activity is expressed as an elevation of maximal response, with little or no enhancement of agonist potency. In terms of Hall's ATSM, this is accomplished by simultaneous positive activation cooperativity and negative binding cooperativity. Most interestingly, amiodarone's PAM activity is readily apparent in the AA response, but not in the IP response. However, when the receptor reserve for the latter response is dramatically reduced, amiodarone's ability to enhance the maximal response of ACh is unmasked. Furthermore, the determination of the magnitude of the receptor reserve allowed for the calculation of agonist affinity (K_A) and demonstrated that ACh has greater affinity for the conformation of the receptor that is associated with the AA response. Finally, the amiodarone analog NEA lacks amiodarone's positive activation cooperativity, but exhibits negative binding cooperativity. The ability of NEA to reverse the PAM effects of amiodarone is consistent with these two ligands competing at a common allosteric site.

4.4 Response equation for the four-ligand allosteric two-state model

4.4.1 Formulation of the response equation

Following the law of conservation of mass, the following equation defines the total population of receptors, $[R]_{total}$ as all of the possible permutations of receptor states that the presence of four ligands can produce:

$$\begin{aligned}[R]_{total} = {} & [R] + [A_1R] + [A_2R] + [RB_1] + [RB_2] \\ & + [A_1RB_1] + [A_1RB_2] + [A_2RB_1] + [A_2RB_2] \\ & + [R^*] + [A_1R^*] + [A_2R^*] + [R^*B_1] + [R^*B_2] \\ & + [A_1R^*B_1] + [A_1R^*B_2] + [A_2R^*B_1] + [A_2R^*B_2]\end{aligned}$$

The parameters in Table 2.1 (p. 43) can be used to substitute terms in the previous equation as:

$$\begin{aligned}[R]_{total} = {} & [R](1 + K_1[A_1] + K_2[A_2] + M_1[B_1] + M_2[B_2] \\ & + \gamma_{11}K_1M_1[A_1][B_1] + \gamma_{12}K_1M_2[A_1][B_2] \\ & + \gamma_{21}K_2M_1[A_2][B_1] + \gamma_{22}K_2M_2[A_2][B_2] \\ & + L(1 + \alpha_1K_1[A_1] + \alpha_2K_2[A_2] + \beta_1M_1[B_1] + \beta_2M_2[B_2] \\ & + \alpha_1\beta_1\gamma_{11}\delta_{11}K_1M_1[A_1][B_1] + \alpha_1\beta_2\gamma_{12}\delta_{12}K_1M_2[A_1][B_2] \\ & + \alpha_2\beta_1\gamma_{21}\delta_{21}K_2M_1[A_2][B_1] + \alpha_2\beta_2\gamma_{22}\delta_{22}K_2M_2[A_2][B_2]))\end{aligned}$$

In the model, response is defined as the sum of all of the active conformations of the receptor, $[R]_{active}$ (R^*, A_1R^*, B_1R^*, etc). A mathematical expression of response can then be defined as the fraction of the total receptor population, $[R]_{total}$, that exists in the active conformation:

$$\frac{[R]_{active}}{[R]_{total}} = \frac{[R^*] + [A_1R^*] + [A_2R^*] + [R^*B_1] + [R^*B_2] + [A_1R^*B_1] + [A_1R^*B_2] + [A_2R^*B_1] + [A_2R^*B_2]}{\begin{aligned}&[R] + [A_1R] + [A_2R] + [RB_1] + [RB_2] + [A_1RB_1] + [A_1RB_2] + [A_2RB_1] + [A_2RB_2] \\ &+ [R^*] + [A_1R^*] + [A_2R^*] + [R^*B_1] + [R^*B_2] + [A_1R^*B_1] + [A_1R^*B_2] + [A_2R^*B_1] + [A_2R^*B_2]\end{aligned}}$$

This equation is also amenable to substitution in a similar manner as above:

$$\frac{[R]_{active}}{[R]_{total}} = \frac{\begin{aligned}&L + \alpha_1K_1L[A_1] + \alpha_2K_2L[A_2] + \beta_1M_1L[B_1] + \beta_2M_2L[B_2] \\ &+ \alpha_1\beta_1\gamma_{11}\delta_{11}K_1LM_1[A_1][B_1] + \alpha_1\beta_2\gamma_{12}\delta_{12}K_1LM_2[A_1][B_2] \\ &+ \alpha_2\beta_1\gamma_{21}\delta_{21}K_2LM_1[A_2][B_1] + \alpha_2\beta_2\gamma_{22}\delta_{22}K_2LM_2[A_2][B_2]\end{aligned}}{\begin{aligned}&1 + K_1[A_1] + K_2[A_2] + M_1[B_1] + M_2[B_2] \\ &+ \gamma_{11}K_1M_1[A_1][B_1] + \gamma_{12}K_1M_2[A_1][B_2] \\ &+ \gamma_{21}K_2M_1[A_2][B_1] + \gamma_{22}K_2M_2[A_2][B_2] \\ &+ L + \alpha_1K_1L[A_1] + \alpha_2K_2L[A_2] + \beta_1M_1L[B_1] + \beta_2M_2L[B_2] \\ &+ \alpha_1\beta_1\gamma_{11}\delta_{11}K_1M_1L[A_1][B_1] + \alpha_1\beta_2\gamma_{12}\delta_{12}K_1M_2L[A_1][B_2] \\ &+ \alpha_2\beta_1\gamma_{21}\delta_{21}K_2M_1L[A_2][B_1] + \alpha_2\beta_2\gamma_{22}\delta_{22}K_2M_2L[A_2][B_2]\end{aligned}}$$

This equation can be further simplified to:

$$\frac{[R]_{active}}{[R]_{total}} = \frac{\begin{array}{c} L(1 + \beta_1 M_1[B_1] + \beta_2 M_2[B_2] \\ + \alpha_1 K_1[A_1](1 + \beta_1\gamma_{11}\delta_{11} M_1[B_1] + \beta_2\gamma_{12}\delta_{12} M_2[B_2]) \\ + \alpha_2 K_2[A_2](1 + \beta_1\gamma_{21}\delta_{21} M_1[B_1] + \beta_2\gamma_{22}\delta_{22} M_2[B_2])) \end{array}}{\begin{array}{c} 1 + M_1[B_1] + M_2[B_2] \\ + K_1[A_1](1 + \gamma_{11} M_1[B_1] + \gamma_{12} M_2[B_2]) \\ + K_2[A_2](1 + \gamma_{21} M_1[B_1] + \gamma_{22} M_2[B_2]) \\ + L(1 + \beta_1 M_1[B_1] + \beta_2 M_2[B_2] \\ + \alpha_1 K_1[A_1](1 + \beta_1\gamma_{11}\delta_{11} M_1[B_1] + \beta_2\gamma_{12}\delta_{12} M_2[B_2]) \\ + \alpha_2 K_2[A_2](1 + \beta_1\gamma_{21}\delta_{21} M_1[B_1] + \beta_2\gamma_{22}\delta_{22} M_2[B_2])) \end{array}}$$

4.4.2 Computational function of the response equation

(for use with the curve-fitting program Graphpad Prism v4.0 and above)

FUNCTIONAL EQUATION

```
A1=10^X;     X is the log of A1
;;;;;;;This section is to assist with setting parameter values in Prism;;;;;;;;;
blankterm0 = A2+B1+B2
K1A1=(K1*A1);     affinity and ligand concentration are always applied together
K2A2=(K2*A2);
blanktermL=L;
M1B1=(M1*B1);
M2B2=(M2*B2);
blankterm1 = alph1+alph2+bet1+bet2+gam11+gam12+gam21+gam22;
blankterm2 = delt11+delt12+delt21+delt22;
;--------------------------------------------------------------------------;
;;;;;;;;;;;;;;;;;;;;;;;;;;;Numerator Definition;;;;;;;;;;;;;;;;;;;;;;;;;;;
numTerm2 = (alph1*K1A1)+(alph2*K2A2);
numTerm3 = (bet1*M1B1)+(bet2*M2B2);
numTerm4 = alph1*K1A1*((bet1*gam11*delt11*M1B1)+(bet2*gam12*delt12*M2B2));
numTerm5 = alph2*K2A2*((bet1*gam21*delt21*M1B1)+(bet2*gam22*delt22*M2B2));
num = L*(1+numTerm2+ numTerm3+numTerm4+numTerm5);
;--------------------------------------------------------------------------;
;;;;;;;;;;;;;;;;;;;;;;;;;;;Denominator Definition;;;;;;;;;;;;;;;;;;;;;;;;;;;
denterm2 = (K1A1+K2A2);
denterm3 = (M1B1+M2B2);
denterm4 = K1A1*((gam11*M1B1)+(gam12*M2B2));
denterm5 = K2A2*((gam21*M1B1)+(gam22*M2B2));
den=(1+denterm2+denterm3+denterm4+denterm5+num);
;--------------------------------------------------------------------------;
Y=num/den;
```

COMMENTS ON FUNCTIONAL EQUATION

PLEASE NOTE: blankterm0, blanktermL, blankterm1, and blankterm2 are only defined to assist when setting parameter values in Prism (that is, they define the order of appearance in the parameter definition table)

Parameters

A1 - concentration of A1 (agonist) [expressed as a function of X]
A2, B1, B2 - concentration of other ligands
K1, K2, M1, M2 - affinity of A1, A2, B1, B2 (respectively)
L – receptor isomerization constant
alph1, alph2 - intrinsic efficacy for A1, A2
bet1, bet2 - intrinsic efficacy for B1, B2
Binding cooperativity between ligands
- gam11 - binding cooperativity between A1 and B1
- gam12 - binding cooperativity between A1 and B2
- gam21 - binding cooperativity between A2 and B1
- gam22 - binding cooperativity between A2 and B2

Activation cooperativity between ligands
- delt11 - activation cooperativity between A1 and B1
- delt12 - activation cooperativity between A1 and B2
- delt21 - activation cooperativity between A2 and B1
- delt22 - activation cooperativity between A2 and B2

4.5 Binding equation for the four-ligand allosteric two-state model

4.5.1 Formulation of the binding equation

The equation that expresses ligand binding is comparable to that used for receptor response in that it identifies a subset of receptor states as those relevant for investigation. The important receptor states in binding experiments are all those labeled with the orthosteric radioligand, A_1 (i.e., A_1R, A_1RB_1, etc). All of the possible states of bound orthosteric radioligand are expressed as:

$$[A_1]_{bound} = [A_1R] + [A_1RB_1] + [A_1RB_2] + [A_1R^*] + [A_1R^*B_1] + [A_1R^*B_2]$$

From this definition of bound receptor forms, the fractional occupancy of the radioligand can be expressed as:

$$\frac{[A_1]_{bound}}{[R]_{total}} = \frac{[A_1R] + [A_1RB_1] + [A_1RB_2] + [A_1R^*] + [A_1R^*B_1] + [A_1R^*B_2]}{\begin{array}{c}[R] + [A_1R] + [A_2R] + [RB_1] + [RB_2] + [A_1RB_1] + [A_1RB_2] + [A_2RB_1] + [A_2RB_2] \\ + [R^*] + [A_1R^*] + [A_2R^*] + [R^*B_1] + [R^*B_2] + [A_1R^*B_1] + [A_1R^*B_2] + [A_2R^*B_1] + [A_2R^*B_2]\end{array}}$$

Utilizing the parameters expressed in Table 2.1 (p. 43), the previous equation can be rewritten as:

$$\frac{[A_1]_{bound}}{[R]_{total}} = \frac{\begin{array}{c}K_1[A_1] + \gamma_{11}K_1[A_1]M_1[B_1] + \gamma_{12}K_1[A_1]M_2[B_2] \\ + \alpha_1K_1[A_1]L + \alpha_1\beta_1\gamma_{11}\delta_{11}K_1[A_1]M_1[B_1]L + \alpha_1\beta_2\gamma_{12}\delta_{12}K_1[A_1]M_2[B_2]L\end{array}}{\begin{array}{c}1 + K_1[A_1] + K_2[A_2] + M_1[B_1] + M_2[B_2] \\ + \gamma_{11}K_1[A_1]M_1[B_1] + \gamma_{12}K_1[A_1]M_2[B_2] \\ + \gamma_{21}K_2[A_2]M_1[B_1] + \gamma_{22}K_2[A_2]M_2[B_2] \\ + L + \alpha_1K_1[A_1]L + \alpha_2K_2[A_2]L + \beta_1M_1[B_1]L + \beta_2M_2[B_2]L \\ + \alpha_1\beta_1\gamma_{11}\delta_{11}K_1[A_1]M_1[B_1]L + \alpha_1\beta_2\gamma_{12}\delta_{12}K_1[A_1]M_2[B_2]L \\ + \alpha_2\beta_1\gamma_{21}\delta_{21}K_2[A_2]M_1[B_1]L + \alpha_2\beta_2\gamma_{22}\delta_{22}K_2[A_2]M_2[B_2]L\end{array}}$$

This equation can be simplified to:

$$\frac{[A_1]_{bound}}{[R]_{total}} = \frac{\begin{array}{c}K_1[A_1](1 + \gamma_{11}M_1[B_1] + \gamma_{12}M_2[B_2] \\ + \alpha_1L(1 + \beta_1\gamma_{11}\delta_{11}M_1[B_1] + \beta_2\gamma_{12}\delta_{12}M_2[B_2]))\end{array}}{\begin{array}{c}1 + K_1[A_1] + K_2[A_2] + M_1[B_1] + M_2[B_2] \\ + K_1[A_1](\gamma_{11}M_1[B_1] + \gamma_{12}M_2[B_2]) \\ + K_2[A_2](\gamma_{21}M_1[B_1] + \gamma_{22}M_2[B_2]) \\ + L(1 + \alpha_1K_1[A_1] + \alpha_2K_2[A_2] + \beta_1M_1[B_1] + \beta_2M_2[B_2] \\ + \alpha_1K_1[A_1](\beta_1\gamma_{11}\delta_{11}M_1[B_1] + \beta_2\gamma_{12}\delta_{12}M_2[B_2]) \\ + \alpha_2K_2[A_2](\beta_1\gamma_{21}\delta_{21}M_1[B_1] + \beta_2\gamma_{22}\delta_{22}M_2[B_2]))\end{array}}$$

4.5.2 Computational function of the binding equation

(for use with the curve-fitting program Graphpad Prism v4.0 and above)

BINDING EQUATION

```
A1=10^X;     X is the log of A1
;;;;;;;This section is to assist with setting constants in Prism;;;;;;;;;
blankterm0=A2+B1+B2;
K1A1=(K1*A1);     affinity and ligand concentration are always applied together
K2A2=(K2*A2);
blanktermL=L;
M1B1=(M1*B1);
M2B2=(M2*B2);
blankterm1=alph1+alph2+bet1+bet2+gam11+gam12+gam21+gam22;
blankterm2=delt11+delt12+delt21+delt22;
;-------------------------------------------------------------------------;
;;;;;;;;;;;;;;;;;;;;;;;;;;;Numerator Definition;;;;;;;;;;;;;;;;;;;;;;;;;;;
numTerm2=((gam11*K1A1*M1B1)+(gam12*K1A1*M2B2));
numTerm3=(alph1*K1A1*L);
numTerm4=(alph1*bet1*gam11*delt11*K1A1*M1B1*L);
numTerm5=(alph1*bet2*gam12*delt12*K1A1*M2B2*L);
num=(K1A1+numTerm2+numTerm3+numTerm4+numTerm5);
;-------------------------------------------------------------------------;
;;;;;;;;;;;;;;;;;;;;;;;;;;;Denominator Definition;;;;;;;;;;;;;;;;;;;;;;;;;;;
denterm6=(K1A1*((gam11*M1B1)+(gam12*M2B2)));
denterm7=(K2A2*((gam21*M1B1)+(gam22*M2B2)));
denpart1=(1+K1A1+K2A2+M1B1+M2B2+denterm6+denterm7);
denTerm8=(alph1*K1A1)+(alph2*K2A2);
denTerm9=(bet1*M1B1)+(bet2*M2B2);
denTerm10=alph1*K1A1*bet1*gam11*delt11*M1B1;
denTerm11=alph1*K1A1*bet2*gam12*delt12*M2B2;
denTerm12=alph2*K2A2*bet1*gam21*delt21*M1B1;
denTerm13=alph2*K2A2*bet2*gam22*delt22*M2B2;
denpart2=(L*(1+denTerm8+denTerm9+denTerm10+denTerm11+denTerm12+denTerm13));
den=denpart1+denpart2;
;-------------------------------------------------------------------------;
Y=num/den;
```

COMMENTS ON BINDING EQUATION

PLEASE NOTE: blankterm0, blanktermL, blankterm1, and blankterm2 are only defined to assist when setting parameter values in Prism (that is, they define the order of appearance in the parameter definition table)

Parameters

A1 - concentration of A1 (radioligand) [expressed as a function of X]
A2, B1, B2 - concentration of other ligands
K1, K2, M1, M2 - affinity of A1, A2, B1, B2 (respectively)
L – receptor isomerization constant
alph1, alph2 - intrinsic efficacy for A1, A2
bet1, bet2 - intrinsic efficacy for B1, B2
Binding cooperativity between ligands
- gam11 - binding cooperativity between A1 and B1
- gam12 - binding cooperativity between A1 and B2
- gam21 - binding cooperativity between A2 and B1
- gam22 - binding cooperativity between A2 and B2

Activation cooperativity between ligands
- delt11 - activation cooperativity between A1 and B1
- delt12 - activation cooperativity between A1 and B2
- delt21 - activation cooperativity between A2 and B1
- delt22 - activation cooperativity between A2 and B2

4.6 Development of the four-ligand allosteric two-state model

The four-ligand allosteric two-state model is an expansion of the cubic allosteric two-state model into a tetra-cubic form (Figure 4.1). In order to demonstrate the construction of the model, we assume that four ligands, two designated as A_x and two designated as B_x, associate with a receptor (as described in Figure 4.10). Each of these ligands can participate in a two-state model, regardless of its binding site (Figure 4.11). If we assume that there are two binding sites, and that ligands designated as A_x associate with one site and ligands designated as B_x associate with the other binding site, we can develop a ternary complex model for each A_x:B_x pair (Figure 4.12). Notice that Figures 4.11 and 4.12 share the ligand association step demonstrated in Figure 4.10; it is possible to express these figures together as Figure 4.13. From Figure 4.13, the further assumption is made that the A_x:B_x pairs are not only able to simultaneously bind, but are also able to stimulate response. We then insert the active state of each bound A_x:B_x pair to develop the four-ligand allosteric two-state model (Figure 4.14).

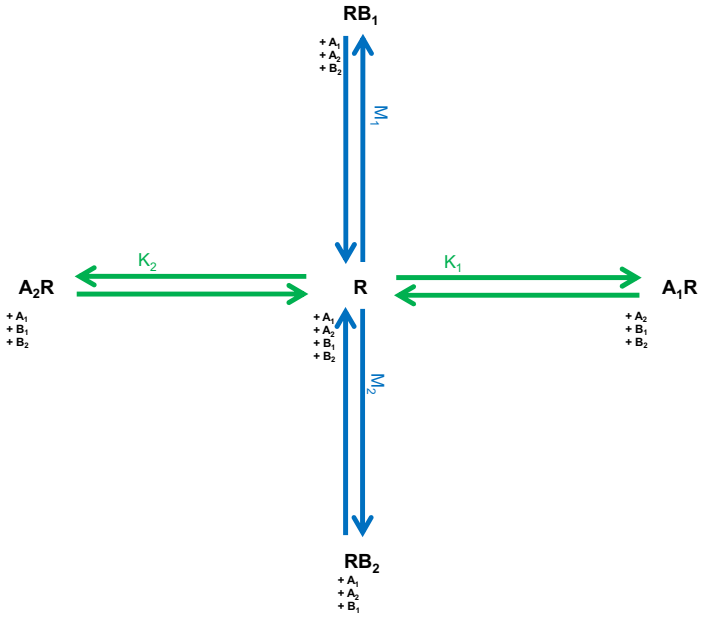


Figure 4.10. Four-ligand association model

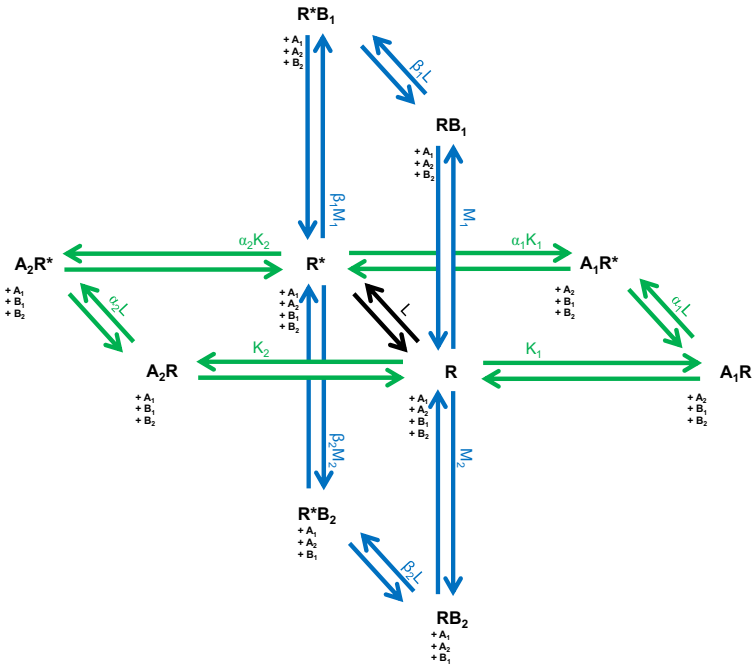


Figure 4.11. Four-ligand two-state model

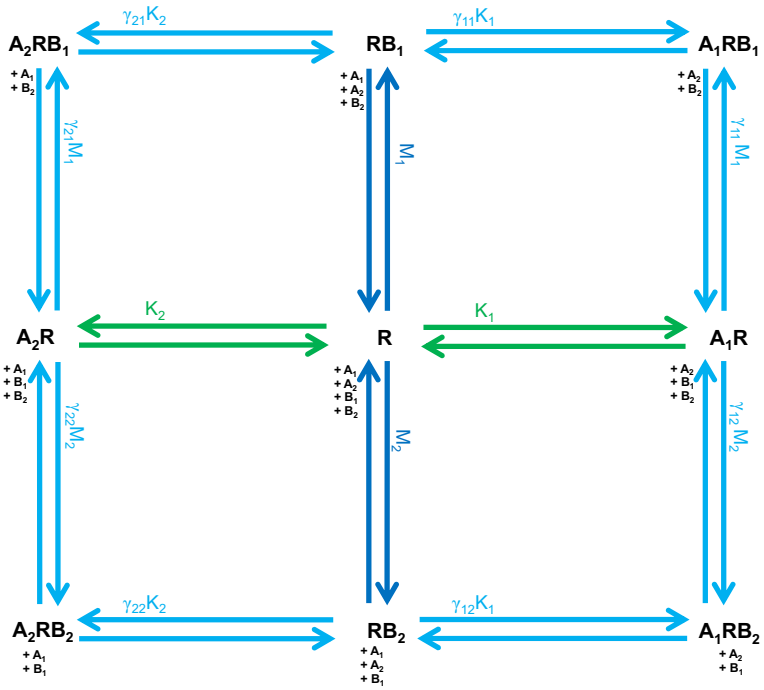


Figure 4.12. Four-ligand ternary complex model

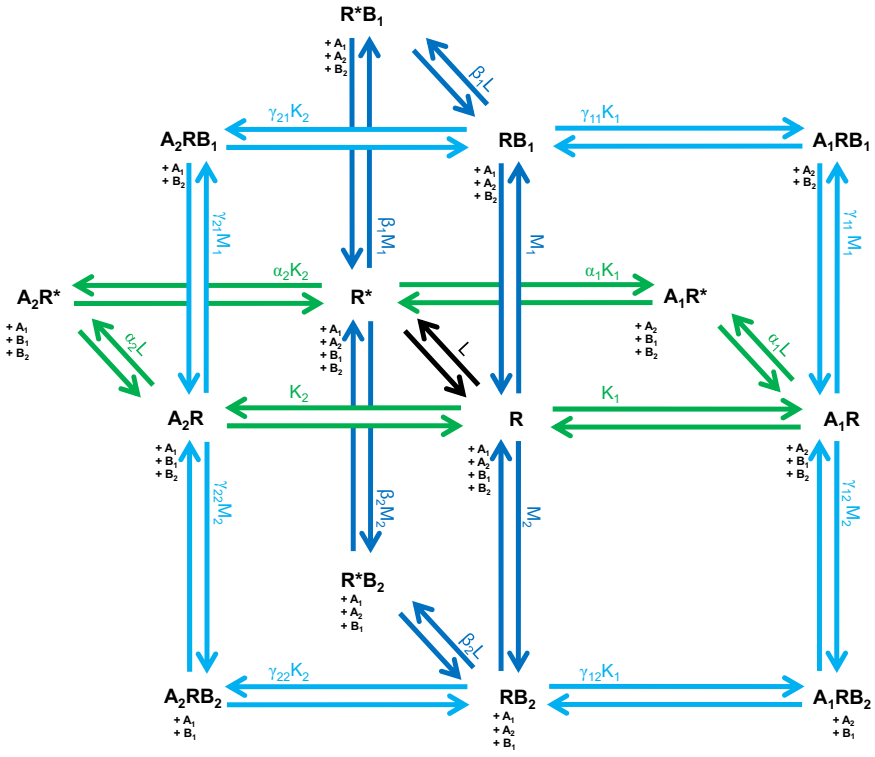


Figure 4.13. Composite model of Figure 4.11 and Figure 4.12

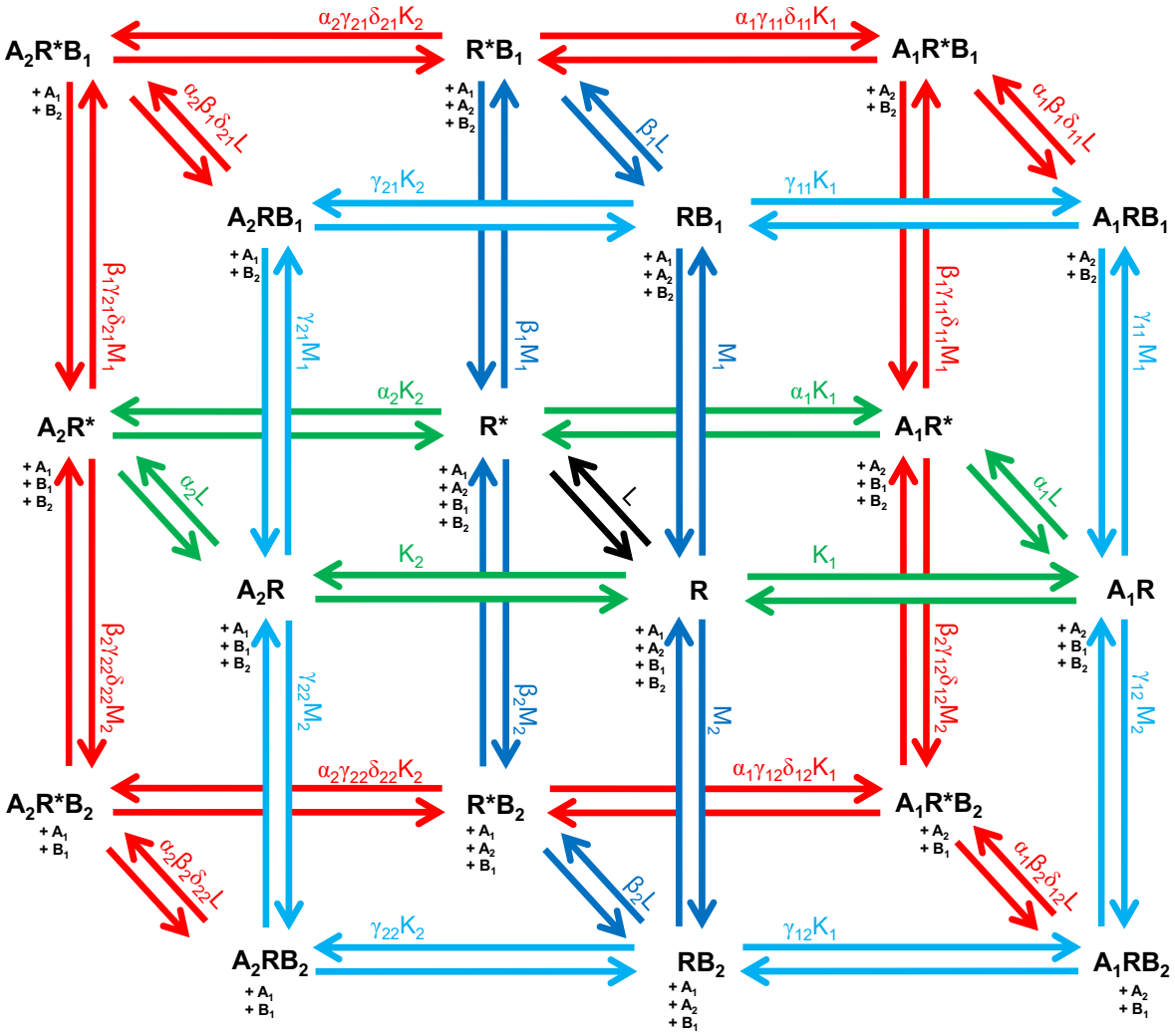


Figure 4.14. The four-ligand allosteric two-state model

Chapter 5

General Discussion

5.1 Discussion

From a historical perspective, the identification of muscarinic receptors occurred in several steps. Almost a century ago, Henry Dale discovered that ACh receptors could be segregated into two classes (Dale, 1914). One class was the nicotinic receptors, named for the ability of nicotine to activate them. The second class of ACh receptors was the muscarinic receptors, named for the ability of muscarine to stimulate their activity. Although the two types of cholinergic receptors could be distinguished, the tools were not available to distinguish between individual muscarinic subtypes.

Hammer et al. (1980) proposed the existence of two muscarinic subtypes, based on the different binding affinities of the antagonist pirenzepine for receptors in the brain and heart. Then, in the mid 1980s, the M_1 and M_2 receptors were cloned. These correlated with the two muscarinic subtypes Hammer et al. had predicted. Subsequently, three more muscarinic receptors were cloned (M_3-M_5). The existence of five different subtypes, all with similar pharmacological characteristics, might appear to be discouraging. However, distinguishing multiple subtypes was actually a fortunate development. The five subtypes have different expression profiles. The M_2 and M_3 receptors are expressed in the periphery where they control numerous functions including smooth muscle tone. These two subtypes are believed to be the cause of many of the undesirable side effects associated with the administration of non-selective muscarinic ligands and AChEIs. All of the muscarinic subtypes are expressed in the CNS. Their expression profile within the CNS is not diffuse. Rather, there are differences in the regions of the brain where individual subtypes are expressed. Due to

these differences in expression, selectively targeting individual subtypes may be beneficial in treating some diseases without producing undesirable side effects.

Our work has focused on investigating the novel allosteric effects of amiodarone. Radioligand binding studies, utilizing both equilibrium and kinetic measures, demonstrated that amiodarone interacts with muscarinic receptors (Figure 3.1). This finding was in partial agreement with previously published work. Cohen-Armon et al. (1984) and Colvin et al. (1989) both demonstrated effects of amiodarone in muscarinic binding studies. However, they concluded that amiodarone was a competitive antagonist. The results of our studies clearly indicated that amiodarone did not compete at the orthosteric ACh binding site. Rather, amiodarone's effect on muscarinic receptor binding was characteristic of a ligand that associates with an allosteric site.

In binding studies that measured radioligand dissociation kinetics we were able to investigate the specific site to which amiodarone associates. Several muscarinic allosteric modulators, including gallamine, have been shown to act at a common binding site using this approach (Ellis and Seidenberg, 1992; Ellis and Seidenberg, 2000). We found that amiodarone and gallamine do not share a common site of action. This suggests that amiodarone associates with a novel allosteric site on muscarinic receptors.

The identification of a novel allosteric site on muscarinic receptors may prove useful for developing novel therapeutic compounds. This fact can be illustrated by discussing $GABA_A$ receptors and the utility of the multiple allosteric sites they possess. γ-aminobutyric acid (GABA) acts as an inhibitory neurotransmitter. The $GABA_A$ receptor is a ligand gated ion channel which allows chloride ions to flow into neurons (Johnston,

1996). This chloride ion flow causes hyperpolarization of the membrane and inhibition of neuronal activity. Along with the GABA binding site, several allosteric sites have been identified on these receptors. Ligands which target these allosteric sites have very useful pharmacological properties and these ligands have been useful in treating a number of conditions.

Barbiturates were used for over half a century as anesthetics and to treat convulsions. They modulate $GABA_A$ receptors through a positive allosteric mechanism of action which prolongs the length of time the channel remains open when GABA is associated (Macdonald et al., 1989). Although barbiturates were quite useful in the past, they possess significant risk of overdose and dependency.

Ethanol is another allosteric modulator of $GABA_A$ receptors (Johnston, 1996). It is believed to bind, with different affinities, to two sites on $GABA_A$ receptors. These sites are distinct from the site of action of the barbiturates. Ethanol causes sedative effects at low concentrations and anesthetic effects at higher concentrations. These two effects are believed to be due to its different affinities for the two sites. Similar to barbiturates, ethanol consumption poses significant risk of overdose and dependency.

Benzodiazepines act at yet another allosteric site on $GABA_A$ receptors. Like barbiturates and ethanol, they are positively cooperative with GABA. They have been extremely useful in the treatment of anxiety, sleep disturbances, seizure, and several other disorders (Johnston, 1996). In contrast to the barbiturates, this class of compounds is more selective for a subset of $GABA_A$ receptors. Benzodiazepine overdose is infrequent and typically involves consumption in combination with alcohol, opioids, or other CNS depressants (Fraser, 1998).

From these examples, a good rationale exists for the continued pursuit of novel allosteric sites not only at $GABA_A$ receptors but also at other neuroreceptors. We have classified the effects of amiodarone on muscarinic receptor binding. As mentioned previously, these studies demonstrated that amiodarone acts at a new allosteric site. In agreement with these binding studies, amiodarone also exhibited allosteric effects on receptor-mediated response. Amiodarone does not possess intrinsic agonist activity. Therefore, if amiodarone did associate with the orthosteric site it would act as a competitive antagonist. We found that amiodarone caused potentiation of agonist-mediated response (as measured by arachidonic acid release). Potentiation of response, without intrinsic agonist activity, indicates that amiodarone associates with an allosteric site, and not the orthosteric site, on the receptor.

As is the case with the $GABA_A$ receptors, some allosteric sites on muscarinic receptors may be more useful than others. The recent discovery of several muscarinic allosteric ligands highlights this point. These ligands bind to an allosteric site on the receptor which is different from the site to which gallamine binds (Spalding et al., 2002). Their association with this allosteric site directly stimulates receptor activity; they act as allosteric agonists. This discovery is interesting because it demonstrates the existence of another allosteric site on the muscarinic receptors. Furthermore, it demonstrates that the allosteric site on the receptor is capable of causing the receptor to be activated. In a similar manner, the allosteric effects of amiodarone on muscarinic receptors provide proof-of-principle. Amiodarone is the first allosteric ligand that causes elevation of maximal response at the M_3 or M_5 subtypes. The effects of amiodarone demonstrate that muscarinic receptors possess an allosteric site which is capable of elevating

maximal response. This indicates that it would be reasonable to pursue other such allosteric modulators which are capable of elevating maximal response.

An allosteric site that causes enhancement of maximal response may have practical applications. Specifically, allosteric ligands that possess this property may be useful in selectively targeting tissues which suffer from significant receptor loss. For example, studies have suggested that, in some cases of schizophrenia, cognitive dysfunction correlated with diminished M_1 receptor expression. Positive allosteric modulators which enhance ACh potency could replace some of the lost cholinergic tone. However, it may be that receptor expression is reduced to the point where activation of all receptors is not capable of producing the necessary stimulation. Positive allosteric modulators which enhance efficacy could increase the signaling potential of the remaining receptors. This effect would be specific for allosteric modulators which enhance efficacy. Agonists with efficacy equivalent to the endogenous neurotransmitter and positive allosteric modulators which enhance potency would not be able to produce stimulation above the level that would result from maximal occupancy of the endogenous neurotransmitter.

The ability of the amiodarone site to potentiate efficacy is a unique effect. Positive allosteric modulation is commonly represented by a leftward shift in response curves and, therefore, an increase in agonist potency. This increase in potency can be expressed mathematically by γ (binding cooperativity) values greater than one in the allosteric two-state model. Detailed investigation of the allosteric effects of amiodarone demonstrated that it does not significantly enhance potency. Instead, amiodarone causes elevation of the maximal response (Figure 3.5). In the allosteric two-state

model, γ values greater than one cannot accommodate the elevation of maximal response, but δ (activation cooperativity) values greater than one can accommodate the elevation of maximal response. Therefore, according to this model, ligands that associate with the amiodarone allosteric site can modulate ACh-mediated response through δ values greater than one (positive activation cooperativity).

Hall (2000) points out that an allosteric modulator with positive activation cooperativity is also predicted to cause a leftward shift in the response curve (an increase in agonist potency). Amiodarone did not cause this predicted leftward shift (Figure 3.5). Rather, ACh potency remained unaffected although maximal response was elevated. Both γ and δ are necessary to describe this effect. γ values less than one cause a rightward shift in potency. γ values less than one, along with δ values greater than one, would have counterbalancing effects resulting in the potency of the agonist remaining unaffected. In this case, the distinguishable characteristic would be the increase in maximal response with little or no effect on agonist potency.

The work published by Price et al. (2005) provided related findings. In this paper, several allosteric modulators were investigated in binding and response studies. In binding studies, the allosteric modulators caused an increase in the radioligand's binding affinity. This finding indicates a specifically allosteric mechanism of action. In response studies, the authors demonstrated that the allosteric ligand caused an enhancement in agonist potency. This increase in potency was accompanied by a decrease in the maximal response. These two effects are opposite to the effects we have observed with amiodarone. However, they illustrate the point that allosteric ligands can have very different effects on agonist potency and efficacy. Other authors

have demonstrated similar effects of allosteric ligands on potency and efficacy (Litschig et al., 1999; Watson et al., 2005).

We also investigated the effects of amiodarone on IP metabolism, another form of muscarinic receptor mediated signaling. In this response, amiodarone caused enhancement of ACh potency but did not elevate maximal response. This was different from the effects of amiodarone on arachidonic acid release where amiodarone elevated the maximal response. We determined that IP metabolism stimulated by M_3 was subject to a significant receptor reserve. (AA release was not subject to a receptor reserve. This is why amiodarone is capable of enhancing maximal AA release.) Upon removal of this receptor reserve, we found that amiodarone was capable of elevating maximal IP metabolism; the receptor reserve masks amiodarone's ability to enhance maximal response. We have speculated that this complication could be a reason why so few allosteric ligands have been found to elevate efficacy. If a receptor reserve is present, and not identified, allosteric enhancement of efficacy might be missed completely or could be interpreted as potency enhancement (as was the case, initially, for amiodarone in modulating IP metabolism at M_3).

In order to determine the degree of receptor reserve, binding and functional experiments were employed. The total number of [^{3}H]NMS binding sites was measured after treatment with increasing concentrations of an irreversible antagonist. The maximal response, for arachidonic acid release as well as IP metabolism, was also measured after treatment with the same concentrations of irreversible antagonist. The results of these binding and response studies were then simultaneously fit to an equation which compares the fraction of response remaining to the fraction of residual

binding sites (Figure 4.6). If the fit to the equation results in a linear relationship between residual binding sites and the remaining response, no receptor reserve is present. However if the equation defines a hyperbolic relationship between residual binding sites and the response remaining, a receptor reserve is present. This is a novel approach for determining receptor reserve and it may be a particularly appealing approach because it is not necessary to know the agonist's affinity or potency.

In fact, this analysis provides a way of determining agonist affinity. The fit of the data to the equation produces a parameter value for the agonist in each response, the *r* value. The *r* value represents the ratio of the agonist's potency to its affinity. The agonist's potency, in both responses, was determined from independent experiments. It was therefore possible to calculate the agonist affinity for the receptor in each response. Interestingly, the affinity of ACh was different for the two responses. This suggests that the receptor adopts different active conformations when stimulating each of the responses.

There is additional evidence in the literature indicating that these responses are the result of different active receptor conformations. Perez et al. (1996) identified a mutation in α_{1B}-adrenergic receptors that induced constitutive activity in stimulating IP metabolism, but did not affect AA release. More closely related to our findings, Berg et al. (1998) observed differences in the affinities of $5HT_{2C}$ agonists for the active states that lead to IP and AA responses in CHO cells. Furthermore, Kurrasch-Orbaugh et al. (2003a) found different magnitudes of receptor reserve for the $5HT_{2A}$ receptors in the two responses. Taken together, these finding suggest that the two pathways are independent and are the result of different active states.

The two-state model postulates that receptors have one active state and one affinity value of the agonist for this active state. It then defines the efficacy of an agonist as the ratio of the affinity of the agonist for the active state to the affinity of the agonist for the inactive state. In this model, the agonist's efficacy would be the same in all forms of response. Therefore, the relative efficacies of multiple agonists in one measure of response would predict their relative efficacies in another measure of response (Kenakin, 2011). Functional selectivity is the characteristic of some ligands to have different efficacies for different responses. In the extreme example, a ligand may be an agonist (greater affinity for the active state) in one measure of response and a neutral antagonist (equivalent affinities for the inactive and active states) in another measure of response. Our finding that agonists have different affinities in different responses supports the work of others on functional selectivity.

Mottola et al. (2002) demonstrated that dihydrexidine is competitive with dopamine D_2 receptor ligands. In functional studies, dihydrexidine exhibited functional selectivity. Dihydrexidine acted as a D_2 agonist on neurons by inhibiting cAMP formation. However, dihydrexidine was found to have little or no agonist activity in other measures of response that D_2 was known to inhibit (e.g. cell firing and dopamine release). These findings demonstrate the physiological significance of functionally selective ligands. Other researchers have also demonstrated functional selectivity (Azzi et al., 2003; reviewed in Kenakin, 2011).

The development of functionally selective drugs may be useful in the clinic. In Alzheimer's disease, M_1-mediated PKC activation may have utility in preventing the development of amyloid plaques. M_1 receptor selective ligands are therefore desirable.

However, activation of M_1 receptors in all forms of response may produce undesirable side effects. It may be possible to develop M_1 selective ligands which preferentially activate PKC without stimulating other forms of response. Langmead et al. (2008b) has raised this possibility with regard to the new class of muscarinic M_1-selective allosteric agonists and their potential in treating Alzheimer's disease.

Our findings demonstrate that the M_3 receptor exhibits different degrees of receptor reserve for arachidonic acid release and inositol phosphate metabolism. Although the M_3 receptor is not particularly attractive as a therapeutic target, the M_1 and M_5 receptor also stimulate both responses. It may be that the M_1 and M_5 receptors stimulate different responses through different active conformations. If this is the case, ligands that are selective for a particular active state could be useful. In order to pursue this functional selectivity there are issues that will need to be addressed. One issue is that ligands which exhibit functional selectivity will need to be developed. Another issue is the need to determine what pathway(s) produce desirable effects in treating different pathologies. Although it will take time to tease out these intricacies, the end result may be ligands which produce much greater therapeutic utility while avoiding many of the complications of drugs with less selective pharmacological profiles. In the same way that the identification of cholinergic receptors led to the discovery of nicotinic and muscarinic receptors, and the identification of muscarinic receptors led to the recognition that there are actually five muscarinic subtypes, the discovery of functionally selective ligands will produce greater complexity. This greater complexity is accompanied by greater opportunity for the selective modulation of receptor signaling.

5.2 The four-ligand allosteric two-state model

The most striking effect of amiodarone is its ability to potentiate agonist mediated maximal response. We have proposed that, according to the allosteric two-state model, this effect is due to δ values greater than one (positive activation cooperativity). The effect of N-ethylamiodarone (NEA) on response is quite different. NEA did not alter maximal response but, similar to a competitive antagonist, caused a rightward shift in response curves. This would suggest that, according to the allosteric two-state model, the effects of NEA on response are due to γ values less than one (negative binding cooperativity).

In order to further determine the mechanism of action of NEA, it was informative to compare the way NEA and the competitive antagonist NMS affected amiodarone potentiation of maximal response. Like NEA, NMS does not potentiate maximal response (Figure 4.8). If either of these ligands occupies the same site as amiodarone then that ligand should reverse amiodarone's effects. We found that NMS did not reverse the potentiation of response caused by amiodarone. However, NEA did reverse this potentiation.

To date, there had not been a response model developed that was able to describe ligand competition at the orthosteric site and the allosteric site. These findings produced the need to develop a new response model that could describe competition at both sites. The development of the response equation is presented in *Section 4.4*. As described in *Section 4.6*, the four-ligand allosteric two-state model was developed to predict the effects of two orthosteric ligands, and two allosteric ligands, on response.

Simulations from the four-ligand allosteric two-state model were generated in order to describe the effects of NEA and NMS on amiodarone potentiation. The model predicts that a neutral antagonist, such as NMS, (A_2 in the model) would have no effect on allosteric potentiation of maximal response by amiodarone. In contrast, the model predicts that an allosteric ligand, such as NEA, (B_2 in the model), would reverse the allosteric potentiation of maximal response (if its activation cooperativity, δ, is equal to one). Specifically the model indicates that the second allosteric ligand, when approaching occupancy at the allosteric site, shifts the equilibrium towards its activation cooperativity. NEA does not enhance or inhibit maximal response and so its activation cooperativity is neutral.

The four-ligand allosteric two-state model describes competition between two allosteric ligands. This is a useful tool for interpreting competition, at an allosteric site, in functional studies. It also makes the distinction between competition at the allosteric site and competition at the orthosteric site. In our studies the model predicted that the site amiodarone and NEA associate with was not the orthosteric site and that amiodarone and NEA were competitive at a common allosteric site. In the same way, the new model would be useful for investigating competition, in functional studies, between other allosteric ligand. It would enable the distinction between competition at the orthosteric site and the allosteric site in response studies.

In recent years, a number of groups have discovered allosteric ligands which elevate efficacy in response (Shirey et al., 2008; Marlo et al., 2009). The new model may be useful for demonstrating that these ligands occupy common binding sites (similar to the effects of amiodarone and NEA) as well as for understanding the

relationship between an allosteric ligand and two orthosteric ligands. It should also be recognized that, along with addressing the functional relationship of two allosteric ligands and two orthosteric ligands, this model could also be used to interpret the effects of these ligands in binding studies. For this reason, the formulation of the binding equation and the computational function of the binding equation are included in *Section 4.5.1 and 4.5.2*, respectively.

5.3 Concluding remarks and future studies

The work presented in this dissertation focuses on understanding the allosteric effects of amiodarone on muscarinic receptors. The predictions of the allosteric two-state model were used to rationalize these findings. As a result, we conclude that amiodarone can elevate maximal response at muscarinic receptors through a novel allosteric site and this site can modulate efficacy. In addition, an elaboration of the allosteric two-state model was developed in order to interpret the effects that ACh, amiodarone, NMS, and NEA exhibited on response. Mathematical modeling using the new model indicated that amiodarone and NEA share a common allosteric site.

The identification of the specific extracellular region(s) of the receptor that compose this site would be very useful information. This may be complicated because a common approach for classifying allosteric sites involves constructing chimeric receptors. The chimeric approach typically employs another receptor subtype, which does not exhibit similar allosteric modulation potential, as a negative control. It is not known, at present, how well conserved this allosteric site is across the entire muscarinic family. If a suitable subtype is not available, other groups have used alanine-scanning mutagenesis as tool for identifying necessary regions of the receptor (Ward et al., 1999). This may be a useful approach; however, it may be hard to distinguish whether the mutation directly disrupts the allosteric site or changes the ability of the receptor to display the site.

Several other muscarinic allosteric ligands have recently been developed. It would be very useful to investigate these newly discovered allosteric ligands, in particular VU0238429. Bridges et al. (2010) identified VU0238429 as the first

muscarinic allosteric ligand that is selective for the M_5 receptor. VU0238429 is believed to act by enhancing the potency of the agonist while not affecting the efficacy of the agonist. VU0238429 and amiodarone may share a common allosteric site and, if so, the allosteric site could be identified using the chimeric receptor approach as VU0238429 is selective for the M_5 receptor.

In the process of investigating the receptor reserve at M_3 it was discovered that the receptor adopts different active conformations when stimulating independent measures of response. It would be useful to investigate the existence of a receptor reserve at the other muscarinic subtypes (and in other measures of response). This may provide a better understanding of the system we are using to study receptor activity and could demonstrate that other allosteric ligands are capable of potentiating efficacy (similar to amiodarone). Furthermore, it may be that investigating the receptor reserve of other subtypes would unmask larger differences in the agonist's affinity for different active conformations of the receptor. If large differences are discovered, this may be useful in understand the physiological significance of these active conformations.

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Curriculum Vitae

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Academic Background

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Scientific Communications (Posters):

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Date: November 15-19, 2008

Society for Neuroscience Annual Meeting, 2009

Amiodarone exerts complex allosteric effects at muscarinic receptors
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Experimental Biology, 2010

Amiodarone and N-ethylamiodarone exert opposing allosteric effects via a novel site on muscarinic receptors
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Publications:

Edward Stahl and John Ellis. Novel allosteric effects of amiodarone at the muscarinic M_5 receptor. Journal of Pharmacology and Experimental Therapeutics 2010, 334(1):214-22.

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