CHARACTERIZATION OF INTERNALIZATION, RECYCLING AND DOWNREGULATION OF MUSCARINIC RECEPTORS

By

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CHARACTERIZATION OF INTERNALIZATION, RECYCLING AND DOWNREGULATION OF MUSCARINIC RECEPTORS

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LIST OF ABBREVIATIONS

4-DAMP	1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide
cAMP	3’-5’-cyclic adenosine monophosphate
FBS	fetal bovine serum
GPCR	G protein-coupled receptor
MDCK	madin darby canine kidney cells
NHERF	Na+/H+ exchange regulatory factor
NSF	N-ethyl-maleimide-sensitive factor
PBS	phosphate buffered saline

CHAPTER I

INTRODUCTION

Of the classes of receptors known, G protein-coupled receptors (GPCRs) are the largest group, constituting more than 800 receptors and GPCRs are the targets for more than 50% of available drugs (Flower, 1999). A wide range of ligands including ions, amino acids, lipid messengers (e.g. anandamide), purines, neuropeptides, peptide hormones, chemokines, glycoprotein hormones, proteases and photons signal through G protein-coupled receptors (Johansen, 2003). The characteristic feature of GPCRs is that they all have seven α-helical transmembrane domains with an extracellular N-terminus, three extracellular loops, three intracellular loops and an intracellular C-terminal tail (Kobilka, 2007). Additionally, they interact with heterotrimeric G proteins (Kobilka, 2007). While the endogenous ligands for several GPCRs are known, the endogenous ligands for a large fraction of the 800 family members have not been identified. These receptors, with no known endogenous ligands, are collectively referred to as orphan receptors (Chung et al., 2008).

1.1 Classification of GPCRs

GPCRs are assigned to one of five families based on sequence similarities within the seven transmembrane domains (Fredriksson, 2003). The five families in the GRAFS system of classification are glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin. Among the five families, the rhodopsin family is the largest containing about 701 members, each having sequence

homology to rhodopsin (Kobilka, 2007). The other families of GPCRs are smaller and do not share sequence similarity with rhodopsin. Receptors for peptide hormones and neuropeptides constitute secretin family, while glutamate family of receptors include $GABA_B$ and metabotropic glutamate receptors (Fredriksson et al., 2003). The receptors studied in this dissertation are muscarinic receptors, belonging to rhodopsin family.

1.1.1 General Characteristics of rhodopsin family members

All receptors in rhodopsin family have characteristic fingerprint residues (Johansen, 2003). An example of the fingerprint residues in family A receptors is the DRY sequence in the third transmembrane domain, which is conserved in greater than 95% of the receptors (Ballesteros et al., 2001). A disulfide bridge between the extracellular loops 1 and 2 is also highly conserved (Palczewski et al., 2000). There are also three conserved proline residues in the transmembrane domains V, VI and VII of rhodopsin family receptors (Johansen, 2003). Conserved proline residues within the alpha helical region may aid in creating different conformations of the receptors when it is bound by a ligand (Palczewski et al., 2000). Lastly, the intracellular loops 2, 3 and the cytoplasmic C tail regions are known to be involved in G protein coupling (Teller et al., 2001).

1.2 Binding sites in GPCRs

GPCRs have both orthosteric and allosteric binding sites, which are described below (Christopoulos and Kenakin, 2002).

1.2.1 Orthosteric binding site

An orthosteric site is a region where endogenous ligands including neurotransmitters, hormones and small molecules bind a receptor (Trumpp-Kallmeyer et al., 1992). When receptors are not bound by a ligand, there is a dynamic equilibrium between active and inactive conformations (Johansen, 2003). Receptor signaling occurs when receptors are in the active

conformation. Consequently, receptors may signal in the absence of a ligand (Tiberi and Caron, 1994). This basal signaling results from a small, but measurable population of receptors that have attained an active state. Agonists are ligands, which bind the receptor at the orthosteric site and stabilize the active conformation of the receptor (Paul, 1995). Agonists have greater inclination to bind to the active conformation of the receptor, increasing the number of receptors in the active state and receptor signaling. When compared to agonists, partial agonists have a lower inclination for the active state of receptors (Johansen, 2003). Hence, the signaling elicited to partial agonists is lower than full agonists acting at the same receptor. Inverse agonists are ligands stabilizing the inactive conformation of the receptor by binding the orthosteric site. They shift the equilibrium towards the inactive state and thereby reduce the basal signaling (Johansen, 2003). Antagonists are ligands, which have inclination to bind the active state and inactive state of receptors equally (Paul, 1995). Therefore, antagonists neither increase nor decrease the signaling of a receptor.

1.2.2 Allosteric binding site

An allosteric site is a binding site distinct from the orthosteric site (Colquhoun, 1998). Allosteric agonists stabilize the active state of receptor by binding the allosteric site (Bridges and Lindsley, 2008). Positive allosteric modulators bind to the allosteric site and enhance the binding of ligands at orthosteric sites (Monod et al., 1965). Negative allosteric modulators decrease the affinity of the orthosteric ligands by binding to allosteric sites (Johansen, 2003). Binding of neutral allosteric ligands to allosteric site have no effect on the orthosteric site binding and just serve to antagonize the binding of other allosteric ligands (Christopoulos and Kenakin, 2002).

In recent times, there is an effort to develop allosteric ligands as they seem promising to achieve selectivity and signaling not achievable with orthosteric ligands (Gregory et al., 2010).

1.3 G protein signaling

GPCRs signal through heterotrimeric G proteins consisting of α, β and γ subunits (Surya et al., 1998; Cabrera-Vera et al., 2003). While α subunits function alone, β and γ subunits

function as heterodimers (Hepler and Gilman, 1992). Alpha subunits have a GTP/GDP binding switch, which serves for activation and deactivation of G protein signaling (Weiss et al., 1988). Agonist binding to GPCRs results in coupling of G proteins to receptors (Ehlert, 1985). The complex of agonist-bound receptor coupled with G protein has a conformation, which favors the exchange of GDP from the Gα subunit for GTP (Christopoulos and Kenakin, 2002). The heterotrimer dissociates from the receptor with the translocation and signaling of Gα and Gβγ subunits through interaction with effector molecules (Neer, 1995). The intrinsic GTPase activity of Gα subunits terminates signaling by hydrolysis of GTP molecule in Gα subunit to GDP (Hepler and Gilman, 1992). G protein signaling is chiefly dependent on the type of Gα associated with Gβγ. Gα subunits are divided into four major groups based on their sequence similarity. $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12/13}$ proteins activate adenylate cyclase, phospholipase C and Rho family of GTPases, respectively (Simon et al., 1991). $G\alpha_i$ proteins inhibit adenylate cyclase enzyme (Woehler and Ponimaskin, 2009).

1.4 History of muscarinic receptors

Mushrooms have been used in various cultures for religious ceremonies for many years. Muscarine was extracted from *Amanita muscaria*, a mushroom used in several religious cultures (Brown, 1989). In frogs, muscarine decreased the heart rate, stopping heart beat at higher concentrations (Brown, 1989). Further, muscarine contracted the smooth muscle of the stomach and intestine and caused secretion of tears, saliva and mucus (Brown, 1989). Acetylcholine, a normal component of animal tissue, also had the same action as muscarine in decreasing heart rate and contracting smooth muscle. In 1921, Loewi performed experiments to prove that the substance released by the vagus nerve is acetylcholine (Brown, 1989). Two frog hearts were used in their study. The vagus nerve leading to one of the frog's heart was stimulated. This slowed down the heart beat. The fluid surrounding the stimulated heart was collected and named as *vagustoff*. When *vagustoff* was introduced into the second frog heart, the heart beat stopped.

While physostigmine, an inhibitor of acetylcholinesterase potentiated the decrease in heart rate caused by *vagustoff*, atropine blocked the decrease in heart rate. *Vagustoff* was unstable in alkali and in serum (Brown, 1989). It was later identified that acetylcholine was the key ingredient in vagustoff. Acetylcholine also activates salivary, lacrimal and sweat glands to release their secretions (Brown, 1989). Acetylcholine is not a circulating hormone and released in the synapse, only in response to the activation of parasympathetic nerves. Acetylcholine mediates various effects such as glandular secretion, decrease in heart rate and contraction of smooth and skeletal muscles by acting through acetylcholine receptors.

Acetylcholine receptors respond to the neurotransmitter acetylcholine and there are two different types: nicotinic and muscarinic acetylcholine receptors. Nicotinic acetylcholine receptors or nicotinic receptors are ligand-gated ion-channel receptors, which respond to nicotine. Nicotinic receptors are present in the central nervous system, peripheral nervous system and in skeletal muscles.

Muscarinic acetylcholine receptors or muscarinic receptors are GPCRs and are the receptors studied in my dissertation. In addition to acetylcholine, muscarinic receptors respond to muscarine and are present both in the central nervous system as well as in the cells innervated by the parasympathetic nervous system. The binding, signaling and localization of muscarinic receptors are described below.

1.5 Characterization of muscarinic receptor subtypes

Muscarinic receptors were purified from porcine brain and heart using affinity chromatography (Haga and Haga, 1983; Peterson et al., 1984). From the purified muscarinic receptors, peptide sequences were obtained to serve as probes for cDNAs encoding full-length muscarinic receptors. Five subtypes of muscarinic receptors were cloned in many species including humans and rats (Kubo et al., 1986a; Kubo et al., 1986b; Bonner et al., 1987; Bonner et al., 1988). Based on the primary sequence of muscarinic receptors, it was predicted that they have

a seven transmembrane spanning domain similar to those identified in rhodopsin and the β_2-adrenergic receptor. Comparison of the sequences of the muscarinic receptor subtypes revealed a significant amount of sequence homology in the transmembrane regions. The greatest variability occurred in the amino and carboxyl termini as well as the third intracellular (i3) loop (Hulme et al., 1990). Five different genes code for the five subtypes of muscarinic receptor and in humans, these muscarinic receptor genes are mapped to chromosomes 11q12-13 (M_1), 7q35-36 (M_2), 1q 43-44 (M_3), 11p12-11.2 (M_4) and 15q26 (M_5) (Caulfield and Birdsall, 1998). Muscarinic receptor genes do not have introns within their coding regions (Caulfield and Birdsall, 1998).

The binding site for acetylcholine in muscarinic receptors is predicted to be near the extracellular region adjoining TM3 with amino acids in TM3, TM6 and TM7 involved in ligand binding (Caulfield and Birdsall, 1998). A very high sequence homology between the muscarinic receptor subtypes around the orthosteric binding site, is the limitation for having subtype-specific ligands for muscarinic receptors (Curtis et al., 1989). Acetylcholine, carbachol, muscarine, pilocarpine, oxotremorine-M, metoclopramide, aceclidine and bethanechol are non-selective muscarinic agonists (Ehlert et al., 1996). McN-A-343, xanomeline and CDD-0097 are M_1 selective agonists. L-689,660 is selective for M_1 and M_3 receptors (Hulme et al., 1990; Caulfield and Birdsall, 1998). Sabcomeline, xanomeline and milameline are partial agonists at muscarinic receptors with no subtype-selectivity (Wood et al., 1999).

The commonly used antagonists, scopolamine, atropine, N-methylscopalamine (NMS) and quinuclidinyl benzilate (QNB) do not distinguish between the different muscarinic receptor subtypes (Hulme et al., 1990; Bolden et al., 1992). AF-DX 116, AF-DX 384, himbacine, methoctramine and tripitramine are selective for M_2 receptors (Eglen et al., 1999). Pirenzepine, telenzepine and muscarinic toxin 7 has higher affinity for M_1 receptors (Eglen et al., 1999). 4-DAMP, p-fluorohexahydro-sila-difenidol and darifenacin are selective for M_3 receptors (Eglen et al., 1996). Tropicamide, muscarinic toxin 3, himbacine and AF-DX 384 have high affinity for M_4

receptors (Eglen et al., 1999). There are no selective antagonists for M_5 receptors (Hulme et al., 1990; Caulfield and Birdsall, 1998).

Because of the high sequence homology around the orthosteric binding site and identification of allosteric binding sites in muscarinic receptors, a number of allosteric ligands were developed. Gallamine was identified as a negative allosteric modulator for M_2 receptors (Ehlert, 1988). Following gallamine, brucine and its derivatives were found to have positive and neutral allosteric modulation at different muscarinic receptor subtypes making them attractive targets for selective action at muscarinic receptor subtypes (Birdsall et al., 1997). Alcuronium and strychnine are positive allosteric modulators for muscarinic receptors (Jakubik et al., 1997). Recently, ML129 was identified as a positive allosteric modulator with selectivity to M_5 receptors (Bridges et al., 2010).

Due to the lack of subtype-specific ligands for different muscarinic receptors, many researchers developed knockout mice. Significant amount of research has been done in the past few decades on muscarinic receptors using radioligands and knockout mice. This led to a deeper understanding of the location of muscarinic receptors in the body, which is described in the subsequent section.

1.6 Distribution of muscarinic receptors

1.6.1 Nervous system

M_1 receptors are the major subtype in the cerebral cortex and hippocampus followed by M_2 and M_4 receptors (Levey, 1993). Additionally, M_1 receptors are expressed in autonomic ganglia. M_2 receptors are predominantly localized in the basal forebrain, thalamus and brainstem regions (Weiner and Brann, 1989; Levey et al., 1991). In the basal forebrain and thalamus, M_3 receptors are expressed to a smaller extent (Levey et al., 1994). M_4 receptors are the major receptors in the striatum along with M_1 and M_2 receptors (Levey et al., 1995). M_5 receptors are localized in the substantia nigra region (Brann et al., 1993).

Muscarinic receptors are localized to both dendrites and axons within neurons. For example, M_1 receptors are localized postsynaptically in the dendrites (Ladner and Lee, 1998; Bartus, 2000; Porter et al., 2002). To the contrary, M_2 and M_4 receptors are localized in the axons of the presynaptic termini of cholinergic neurons, which release acetylcholine into the synapse. This suggests the role of M_2 receptors as autoreceptors inhibiting the release of acetylcholine in cholinergic neurons as well as the release of other neurotransmitters in non-cholinergic neurons (D'Agostino et al., 1997; Iannazzo and Majewski, 2000; Zhang et al., 2002). M_4 receptors are localized in the dendrites of the postsynaptic neurons in the striatum. M_1 and M_4 receptors are also found on the axons presynaptically in some asymmetrical excitatory synapses in the striatum (Hersch and Levey, 1995).

M current is a voltage-dependent K^+ current in the sympathetic ganglion neurons and in hippocampal pyramidal neurons. Activation of muscarinic receptors by muscarinic agonists ablates this current and causes depolarization of plasma membrane. In M_1 knockout mice, muscarinic receptor agonist did not mediate the ablation of M current. Further, muscarinic agonists induce the formation of seizures in wild-type mouse. Additionally, M_1 knockout mice were resistant to the muscarinic agonist-induced seizures, suggesting the role of M_1 receptors in the ablation of M current and initiation of epileptic seizures induced by muscarinic agonists (Hamilton et al., 1997). Activation of muscarinic receptors by muscarine is responsible for hippocampal γ oscillations. Hippocampal γ oscillations stimulated by muscarine were completely abrogated in the M_1 knockout mice, whereas knockout mice of M_2, M_3, M_4 or M_5 had hippocampal γ oscillations comparable to wild-type (Fisahn et al., 2002). M_1 and M_3 receptors were also shown to activate mitogen activated protein kinases (MAPKs). The MAPK pathway in the cortex and hippocampus has been shown to be important in learning and memory, amyloid protein processing, and neuronal plasticity (Hamilton and Nathanson, 2001). Activation of ERK 1/2 protein was significantly impaired in the M_1 knockout mice as well as in PC12 cells treated with a toxin specific for M_1 receptor compared to wild-type mice. This suggests that the M_1

receptor is the key receptor involved in MAPK activation in the mouse forebrain (Berkeley and Levey, 2000).

1.6.2 Eye

Muscarinic receptors are expressed in the eye and mediate contraction of the iris sphincter and ciliary body. M_3 receptors are the predominant subtype of receptor expressed in both the iris sphincter and ciliary body and are known to mediate contraction (Zhang et al., 1995). Contraction of the iris leads to pupillary constriction while contraction of the ciliary body leads to accommodation. Contraction of the ciliary muscle also opens the trabecular network in the angle of the eye, facilitating the draining of aqueous humor. This particular response is useful in the treatment of glaucoma, a disease in which there is an increase in intraocular pressure. Muscarinic agonists (carbachol and pilocarpine) used in the treatment of glaucoma cause an increase in the outflow of aqueous humor and a reduction in intraocular pressure.

M_1, M_2, M_4 and M_5 receptors are also expressed in the eye, though to a lesser extent than M_3. M_1, M_2 and M_4 receptors are expressed in the iris, whereas M_5 is expressed in the ciliary body (Gil et al., 1997).

1.6.3 Salivary glands

In the submaxillary gland, M_3 and M_1 receptors are predominately expressed along with fewer M_2 receptors (Maeda et al., 1988; Dorje et al., 1991; Levey, 1993). Autoantibodies to M_3 muscarinic receptors were produced in patients with the rare autoimmune disease Sjogren's syndrome (Waterman et al., 2000). These autoantibodies act as antagonists at M_3 receptors expressed on salivary glands, inhibiting the secretion of saliva resulting in a condition of dry mouth called called xerostomia. M_3 receptor agonists are used to induce salivation in patients experiencing dry mouth.

1.6.4 Heart

Muscarinic receptors are expressed in the heart and they decrease the heart rate in response to parasympathetic stimulation. M_2 receptors are the major subtype of muscarinic receptors expressed in the heart (Maeda et al., 1988; Levey, 1993). Atria contain only M_2 receptors (Dorje et al., 1991). In the ventricles apart from M_2 receptors, trace amounts of M_3 and M_5 receptors are present (Krejci and Tucek, 2002; Myslivecek et al., 2008). M_2 receptors couple with G_i proteins. In cardiac atrial myocytes, acetylcholine activation of M_2 receptors causes the $G\beta\gamma$ subunit to activate inwardly rectifying K^+ channels (K_{ir}) (Yamada et al., 1998). This causes the channel to open and release K^+ ions outside the cell and cause hyperpolarization of the cells (Yamada et al., 1998). Thereby, M_2 receptors decreases the depolarization caused by sympathetic stimulation of cardiac cells.

1.6.5 Lungs

Muscarinic receptors are expressed in the lungs and they mediate contraction of airway smooth muscles and secretion of mucus. M_1 and M_3 receptors are expressed in the airway glands (White, 1995), while M_2 and M_3 receptor are the prominent subtypes expressed in the airway smooth muscle. Smooth muscle contraction in lungs are mediated by M_3 receptors (Esqueda et al., 1996). Apart from M_3 receptors, M_2 receptors also mediate contraction of smooth muscles indirectly by opposing the relaxation produced by β_2-adrenergic receptors (Proskocil and Fryer, 2005). Airway hyperreactivity is a condition, where the neuronal/ inhibitory M_2 receptors are not functional leading to increased acetylcholine in the synapse and hence increased smooth muscle contractility. In patients suffering from inflammatory conditions such as asthma, eosinophil major basic protein and eosinophil peroxidase are identified to act as an endogenous allosteric antagonist for M_2 receptor mediating airway hyperreactivity (Jacoby et al., 1993). As a result of antagonism of M_2 receptors, the inhibition of synaptic acetylcholine release by M_2 autoreceptors is lost and increased smooth muscle contractility happens through acetylcholine acting on M_3

receptors in the airway smooth muscles. M_3 receptor selective antagonists like ipratropium or tiotropium are used in the treatment of chronic obstructive pulmonary disease (COPD).

1.6.6 Stomach

Muscarinic receptors expressed in stomach are responsible for acid secretion. In stomach, there are three different gastric cells - the enterochromaffin like (ECL) cells producing histamine, the G cells secreting gastrin and the D cells producing somatostatin, all of which control gastric acid secretion. Muscarinic receptors are expressed on all these cells as well as on the parietal cells secreting hydrochloric acid. For example, M_3 receptors are present on the parietal cells along with some M_5 receptors (Aihara et al., 2005). Further, M_1 receptors are expressed in ECL cells, regulating histamine release. Additionally, M_2 and M_4 receptors are present on the D cells (Tobin et al., 2009) and activation of muscarinic receptors in D cells inhibit the release of somatostatin. Muscarinic receptor activation in the gastric cells results in secretion of gastrin. Histamine and gastrin facilitate acid secretion from the parietal cells. To the contrary, somatostatin inhibits acid secretion.

1.6.7 Gastrointestinal tract

In the ileum, two subtypes of muscarinic receptors are expressed and they mediate gastrointestinal motility through smooth muscle contraction (Eglen, 1996). M_2 receptors are the major fraction of muscarinic receptors in the ileum while M_3 receptors represent the minor fraction (Candell et al., 1990). M_3 receptor activation contracts smooth muscles directly, through calcium release. On the other hand, activation of M_2 receptor has an opposing effect on the relaxation produced by other agents that increase the cAMP levels (Ehlert et al., 1999). Since M_3 receptor's contractile action in the smooth muscles are dominant, antagonists for M_3 receptors are used for treatment of irritable bowel syndrome.

1.6.8 Urinary bladder

M_1, M_2, M_3 and M_4 receptors are expressed in the urinary bladder (Abrams et al., 2006). Contraction mediated by M_3 receptors present on the detrusor muscle is responsible for micturition (Matsui et al., 2000). M_2 receptors present on the detrusor muscle indirectly mediate contraction by decreasing cAMP production. M_3 receptor antagonists are used to treat overactive bladder (Abrams et al., 2006).

1.7 Muscarinic receptor signaling

M_1, M_3 and M_5 receptors couple with $G_{q/11}$ proteins and activate phospholipase C (Hammer, 1980; Peralta et al., 1988). Activated phospholipase C catalyzes the breakdown of phosphoinositide 4, 5 bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol. IP_3 activates IP_3 receptors in the endoplasmic reticulum and releases Ca^{2+} from the endoplasmic reticulum. Diacylglycerol along with Ca^{2+} activates protein kinase C, which phosphorylates the receptor and tertiary messenger proteins. Released Ca^{2+} also activates calcium/calmodulin dependent kinases including myosin light chain kinase (MLCK). In turn, MLCK phosphorylates myosin, which causes smooth muscles to contract. Through this mechanism, smooth muscle contraction in gastrointestinal tract and lungs are mediated by M_3 receptors. In vascular smooth muscles, activated calcium/calmodulin dependent kinases activate nitric oxide synthase (NOS) (Furchgott, 1999). NOS converts L-arginine to nitric oxide (NO). NO diffuses across the vascular smooth muscle and activates guanylyl cyclase resulting in the production of cGMP, which mediates vasodilation in the arteries (Furchgott, 1999). Apart from G_q mediated signaling, M_3 receptors are also shown to remodel the actin cytoskeleton by signaling through the small G proteins Rho and cdc42 in SH-SY5Y cells (Linseman et al., 2000).

M_2 and M_4 receptors couple with $G_{i/o}$ proteins and inhibit adenylate cyclase (Parker et al., 1991; Migeon et al., 1995). Thereby M_2 and M_4 receptors decrease cAMP production (Kashihara et al., 1992).

1.8 Regulation of GPCR signaling

The extent and duration of agonist-dependent GPCR signaling is highly regulated. This regulation occurs in four steps: desensitization, internalization and recycling or downregulation which is described below (Ferguson, 2001).

1.8.1 Desensitization

Prolonged exposure to agonists desensitizes receptors. Desensitization is defined as loss of response (receptor signaling) in the continued presence of agonist (Ferguson et al., 1998). The entire process of GPCR desensitization happens in seconds to minutes (Waugh et al., 1999). Desensitization involves phosphorylation of specific serine and threonine residues in the cytoplasmic regions (third intracellular loop and/or C-terminus) of the receptor and uncoupling of the receptors from G proteins (Lefkowitz, 1998). Phosphorylation of agonist-activated GPCRs can be mediated by G protein-coupled receptor kinases (GRKs), second messenger-dependent protein kinases (protein kinases A and C) or casein kinases (Ferguson, 2001). GRKs are specific and target only activated GPCRs when compared to other kinases, which phosphorylate GPCRs in a heterologous manner (Benovic et al., 1986; Lee and Fraser, 1993). Seven different GRKs are expressed in mammals (Premont and Gainetdinov, 2007). GRKs 1 and 7 are visual GRKs and are expressed in the eyes. GRK 4 is also expressed tissue specifically in testis. To the contrary, GRKs 2, 3, 5 and 6 are widely distributed and expressed in all tissues. While GRKs 4, 5 and 6 are localized to the plasma membrane, GRKs 2 and 3 are cytosolic and associate with Gβγ subunits to phosphorylate activated receptors (Pitcher et al., 1992).

Once phosphorylated, regions in GPCRs are bound by arrestin molecules, and arrestins prevent the receptors to couple from G proteins (Luttrell and Lefkowitz, 2002). In mammalian cells, there are four arrestins numbered from 1-4 (Premont and Gainetdinov, 2007). Arrestins 1 and 4 are visual arrestins and have a limited distribution in the eyes. However, arrestins 2 and 3 also represented as β-arrestins 1 and 2 are widely expressed in all tissues and are responsible for

regulation of non-visual GPCR signaling (Attramadal et al., 1992). Following desensitization, agonist-activated GPCRs undergo internalization.

1.8.2 Internalization

Internalization causes GPCRs to be removed from the plasma membrane in vesicles and transported to endosomes. Agonist-activated GPCR internalization can be characterized using membrane permeable and membrane impermeable radioligands. Alternatively, using density-gradient centrifugation, receptors in the heavy fraction (plasma membrane) and light fraction (endosomes) can be quantified. Internalization of GPCRs occurs within minutes to hours of exposure to agonist.

Agonist-bound receptors internalize using different mechanisms, and each mechanism relies on a specific interaction between a receptor domain and endocytic partners. Several motifs present in the cytoplasmic regions are implicated in internalization of GPCRs. These include tyrosine and dileucine based motifs (Gabilondo et al., 1997). Further, phosphorylation of key serine and threonine residues in the cytoplasmic domain serves as internalization determinants for several GPCRs (Moro et al., 1993; Tsuga et al., 1994). Additionally, ubiquitination of lysine residues also serve as an internalization signal for several GPCRs (Wolfe et al., 2007).

A majority of GPCRs undergo internalization by clathrin-mediated endocytosis. In this mechanism, agonist-activated GPCRs are phosphorylated by GRKs and the phosphorylation sites act as binding sites for β-arrestins. Clathrin molecules are recruited by β-arrestin molecules to form a clathrin coated pit, trapping the receptor (Lefkowitz, 1998; Claing et al., 2002). This pit invaginates forming a vesicle and then pinched off by recruitment of dynamin GTPase molecules. Alternatively, some GPCRs can internalize in a β-arrestin-independent manner. AP-2 adaptor proteins can bind directly to both clathrin and a tyrosine based motif in GPCR, resulting in clathrin-mediated internalization. Apart from clathrin-mediated internalization, caveolins can mediate internalization of some GPCRs (Shmuel et al., 2007). Regardless of the internalization

mechanisms (clathrin or caveolin), dynamin GTPases are important for the scission of the vesicles derived from clathrin and caveolin-dependent internalizations (Dessy et al., 2000; Gaborik et al., 2001). Following internalization, the internalized receptors can either recycle back to the plasma membrane in a resensitized state or be transported to the lysosomes and degraded. Whether a receptor recycles or degrades is determined by the sequences present in the receptor.

1.8.3 Recycling

Recycling refers to the trafficking of internalized receptors from endosomes back to the plasma membrane in a resensitized state. There are two major pathways of receptor recycling from endosomes: default recycling (Mayor et al., 1993; Maxfield and McGraw, 2004) or the targeted recycling pathway (Gage et al., 2001; Gage et al., 2005; Paasche et al., 2005). Default recycling occurs through the fusion of endosomes with the plasma membrane by bulk membrane flow and occurs most of the time when the targeted delivery to particular compartments is impaired (Hanyaloglu and Zastrow, 2008). Targeted recycling occurs through specific recycling sequences or motifs, which interact with motor and effector proteins and traffic the receptor containing endosomes back to the plasma membrane (Onoprishvili et al., 2003; Seck et al., 2003; Gardner et al., 2007). For example, the rapid recycling of β_2-adrenergic receptors is dependent on the interaction of sequences in the C-terminus with proteins NHERF-1, NHERF-2, PDZK1 and NSF (Fan et al., 2001). When this sequence was mutated, recycling was aborted and the receptor was directed to lysosomes. Delta opioid receptors typically undergo degradation after agonist-dependent internalization (Gage et al., 2001). When the recycling sequence of the β_2 adrenergic receptor was conjugated to the C-termini of δ-opioid receptors, they recycled to the plasma membrane (Gage et al., 2001). This suggests the importance of the recycling sequence in targeting receptors to the plasma membrane.

1.8.4 Downregulation

Downregulation of GPCRs occurs following prolonged stimulation with agonists. While downregulation can mean reduction in transcription of GPCRs, we are interested in the downregulation at the protein level, which can be defined using membrane-permeate radioligands. A highly permeant radioligand for a GPCR will be a valuable tool in quantitating the total number of receptors expressed in a cell (intracellular and cell surface receptors). Intact, whole cell radioligand binding assay with a highly permeant radioligand allow one to characterize the reduction in receptor number, resulting from incubation of agonist over time. Majority of GPCRs are downregulated in lysosomes (Tsao and von Zastrow, 2000).

1.8.4.1 Lysosomal downregulation

Lysosomes are the primary degradative organelles of the cell. Proteins for degradation in lysosomes are received through endocytosis, phagocytosis and autophagy. Lysosomal hydrolases are enzymes, which target substrates for lysosomal degradation and require acidic pH for their enzymatic activity (Saftig and Klumperman, 2009). For GPCRs downregulated in lysosomes, ubiquitination has been a pre-determinant (Marchese et al., 2008). The predominant mechanism for targeting GPCRs to lysosomes involves trafficking of ubiquitinated receptors to intralumenal vesicles called multivesicular bodies (MVB), which fuse with lysosomes. The sequestration of ubiquitinated GPCRs in MVB relies on an endosomal-sorting complex required for transport (ESCRT) machinery (Wollert et al., 2009). Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) recognizes monoubiquitinated or lysine 63 polyubiquitinated GPCRs in the endosomes (Haglund and Dikic, 2005). Clathrin lattices are formed in the regions of endosomes concentrated with ubiquitinated receptors. ESCRT1, ESCRT 2 and ESCRT 3 proteins are sequentially recruited by HRS and the ubiquitinated receptors are transported to multivesicular bodies, which fuse with lysosomes (Marchese et al., 2008).

Apart from the ubiquitin and ESCRT-dependent pathways of degradation, an ubiquitin-independent pathway also exists (Tanowitz and Von Zastrow, 2002). For example, δ-opioid receptors normally undergo ubiquitin and ESCRT-dependent lysosomal targeting. When all the cytoplasmic lysine residues of δ-opioid receptors are mutated to arginine, they still underwent agonist-promoted downregulation similar to the wild-type receptor indicating the presence of ubiquitin-independent downregulation in mutant δ-opioid receptors (Tanowitz and Von Zastrow, 2002). The mutant receptor was shown to be bound by HRS and GPCR associated sorting protein (GASP) for its delivery to lysosomes independent of ESCRT machinery and ubiquitin (Whistler et al., 2002). Protease-activated receptor 1 (PAR1) also undergoes lysosomal degradation independent of ubiquitin and ESCRT machinery (Gullapalli et al., 2006). Sorting nexin 1, a protein, which localizes to early endosomes, was shown to be involved in the delivery of PAR1 receptors to the lysosomes (Worby and Dixon, 2002).

Upon prolonged agonist-exposure, most GPCRs are targeted to the lysosomes for proteolytic degradation (Shenoy, 2007; Marchese et al., 2008). For example, GFP tagged CXCR4 receptors colocalize with the lysosomal marker LAMP1 after agonist stimulation in HEK 293 cells (Marchese and Benovic, 2001). Similarly, agonist-stimulated β_2-adrenergic receptor was ubiquitinated by the E3 ligase Nedd4, which targets it to the lysosomes for degradation (Shenoy et al., 2008). While the vast majority of GPCRs are targeted to lysosomes for degradation after agonist-stimulation, a small fraction of GPCRs are not degraded in the lysosomes. This is highlighted by the fact that lysosomal inhibitors had little or no effect on the agonist-induced downregulation of μ opioid receptors and histamine H_1 receptors (Chaturvedi et al., 2001; Hishinuma et al., 2010). To the contrary, the downregulation of platelet activating factor receptor (PAFR) is different and takes place both in the proteasomes and lysosomes as specific lysosomal protease and proteasomal inhibitors decreased the agonist-induced downregulation of PAFR (Dupre et al., 2003). Overexpression of dominant negative Rab7, a protein involved in the trafficking of cargo from early endosomes to late endosomes, inhibited the downregulation of

PAFR confirming the trafficking of PAFR to lysosomes (Dupre et al., 2003). These findings suggest that degradation of some GPCRs may take place in proteasomes.

1.8.4.2 Proteasomal downregulation

Proteasomes are protein complexes composed of two subunits: a 19S regulatory subunit and a 20S catalytic subunit together forming a 26S complex (Murata et al., 2009). The 20S proteasome is a cylindrical unit composed of four heptameric rings: two outer alpha rings and two inner beta rings. The alpha rings serve to regulate the entry of proteins into the beta rings and the beta rings have the catalytic activity. The major classes of enzymes present in the beta rings are caspase-like, trypsin-like and chymotrypsin-like (Myung et al., 2001). The 19S regulatory subunit consists of a lid and a base attached to the 20S catalytic subunit. The base contains six ATPases and three non-ATPases proteins, while the lid contains nine non-ATPase proteins. The lid is responsible for identification of polyubiquitinated proteins and deubiquitination (Murata et al., 2009). The deubiquitnated proteins are unfolded and then threaded into the 20S alpha rings by the ATPase proteins in the 19S base (Glickman and Ciechanover, 2002).

1.8.5 Role of Rab proteins in trafficking of GPCRs

Trafficking of proteins between intracellular organelles is regulated by small GTP proteins called Rabs (Jordens et al., 2005). Rab proteins belong to the Ras GTPase superfamily (Martinez and Goud, 1998). More than 60 Rab proteins have been identified in mammalian cells and they are involved in the transport of proteins from one intracellular compartment to another. The various steps involved in Rab-mediated transport of proteins involve selection of cargo, transport of cargo in vesicles, delivery of vesicles to target compartment via molecular motors along the microtubule or actin filament systems, and fusion of the vesicle with the target membrane (Hammer and Wu, 2002; Grosshans et al., 2006).

Lipid modification of cysteine residues of Rabs anchors them to membranes. The lipid anchor is concealed by a GDP displacement inhibitor protein (GDI). A GDI displacement factor

protein (GDF) displaces the GDI and facilitates the membrane insertion of Rab proteins. Rabs are activated by guanine nucleotide exchange factors (GEF), which catalyzes the exchange of GDP to GTP. Rabs cycle between GTP-bound and GDP-bound states. Following activation of Rabs, effector proteins bind and the vesicles are transported. Once the vesicle fuses with membrane of the target organelle, GTP in active Rabs are hydrolyzed by specific GTPase activator proteins (GAP) (Grosshans et al., 2006). The most commonly studied Rabs in trafficking of GPCRs are Rab1, Rab2, Rab3, Rab4, Rab5, Rab7 and Rab11. Rab1 is involved in the transport of vesicles from endoplasmic reticulum to the golgi, while Rab2 is implicated in the retrograde transport of vesicles from the golgi to the endoplasmic reticulum (Zerial and McBride, 2001). Rab3 proteins are engaged in the secretion of proteins or exocytosis (Stenmark, 2009). Rab4 proteins are involved in the transport of vesicles from sorting endosomes to the plasma membrane in the fast recycling pathway, while Rab11 proteins mediate the slow recycling of proteins from the sorting endosomes to the plasma membrane via the perinuclear recycling compartment (Ullrich et al., 1996; Innamorati et al., 2001). Rab5 is involved in both endocytosis of receptors from the plasma membrane to early endosomes and for endosome-endosome fusion (Seachrist et al., 2002). Rab7 proteins are involved in the transport of proteins from sorting endosomes to late endosomes, which then mature into lysosomes (Zerial and McBride, 2001).

1.8.6 Muscarinic receptor trafficking

Regulation of muscarinic receptor signaling follows the same steps of regulation of GPCR signaling as described above.

Desensitization of muscarinic receptors includes phosphorylation of the receptors by kinases and binding of β-arrestin. The desensitization of muscarinic receptors takes place within minutes of exposure to agonists (Tobin et al., 1992; Waugh et al., 1999). Agonist-stimulated desensitization of muscarinic receptors is initiated by the activity of a GRK, which phosphorylates the receptor (van Koppen and Kaiser, 2003). The phosphorylated receptor is then

bound by β-arrestin molecules (Vogler et al., 1999). Bound β-arrestin sequesters the receptor from coupling with G proteins and thus ceases receptor signaling. Beta-arrestin recruits AP2 and clathrin to remove the receptor from the plasma membrane via clathrin-mediated endocytosis (Claing et al., 2002). The clathrin coated pit matures to form a vesicle, which is excised from the plasma membrane by dynamin GTPase (van Koppen, 2001).

M_1, M_3, M_4 and M_5 receptors are known to follow the above mentioned pathway (Tolbert and Lameh, 1996; Tsuga et al., 1998b; Vogler et al., 1999). Expression of a dominant negative β-arrestin mutant in HEK293 cells decreased the internalization of M_1, M_3 and M_4 receptors with no significant effect on the internalization of M_2 receptors. Similarly, expression of dominant negative clathrin hub mutant inhibited the internalization of M_1, M_3 and M_4 receptors with no change in the internalization of M_2 receptors in HEK293 cells (Vogler et al., 1999). In mouse embryonic fibroblasts (MEF), M_2 receptors have been shown to internalize in a β-arrestin and clathrin-dependent fashion (Jones et al., 2006). This suggests that M_2 receptors internalize in a β-arrestin, clathrin-independent and clathrin-dependent fashion in HEK293 cells and MEF cells respectively. Additionally, Rab5 proteins have been implicated in the internalization of muscarinic receptors. For example, internalization of M_4 receptors is regulated by Rab5 (Volpicelli et al., 2001).

Following agonist-induced internalization, recycling of muscarinic receptors takes place. For example, after internalization, M_4 receptors are localized in perinuclear endosomes along with Rab11 from where they slowly recycle to the plasma membrane (Volpicelli et al., 2002). In addition, elongation factor 1A was shown to enhance recycling of M_4 receptors following agonist-induced internalization (McClatchy et al., 2006). M_1, M_2 and M_3 receptors recycle following agonist-induced internalization (Koenig and Edwardson, 1996; Roseberry and Hosey, 1999; Shmuel et al., 2007). Previously, the recycling of M_5 receptors was not characterized.

Agonist-induced downregulation of muscarinic receptors takes place after prolonged incubation with muscarinic receptor agonists. The downregulation of M_1, M_2, M_3 and M_4

receptors have been characterized in different cell types including U293, CHO, HEK293 and At-T20 cells (Lameh et al., 1992; Lenz et al., 1994; Roseberry and Hosey, 1999; Shockley et al., 1999). Previously, the agonist-induced downregulation of M_5 receptors have not been studied.

Although, there is a lot known about the signaling and trafficking of muscarinic receptors, there are still certain unanswered questions. In the studies presented here, a comparison of the trafficking of the muscarinic receptors is explored. Further, the mechanism of agonist-induced downregulation of muscarinic receptors is studied. Moreover, we evaluated the roles of the i3 loop and C-terminal tail in the trafficking of M_1 muscarinic receptors.

1.9 Aims

The primary goal of our research was to understand the trafficking of muscarinic acetylcholine receptors. To accomplish this goal, the following specific aims were pursued.

1.9.1 Characterization of internalization, recycling and downregulation of muscarinic receptors

Subtype- and cell-specific differences have been observed in agonist-dependent processes (desensitization, internalization, recycling and downregulation) of muscarinic receptors expressed in endogenous and heterologous cell lines. For example, M_1 and M_3 receptors internalized to a lesser extent than M_2 and M_4 receptors in CHO and COS7 cells in response to carbachol-treatment (Koenig and Edwardson, 1996; Tsuga et al., 1998b). The rates of carbachol-induced internalization were significantly higher in SH-SY5Y neuroblastoma cells endogenously expressing M_3 receptors when compared to CHO cells stably transfected with M_3 receptors (Koenig and Edwardson, 1996). These are a few of many examples of subtype- and cell-specific differences in the agonist-dependent regulation of muscarinic receptors. While sequence differences among subtypes could account for subtype-specific differences, cell-specific differences are attributed to differences in the expression of proteins involved in trafficking between endogenous and heterologous cell lines. For example, β-arrestin 2, a protein involved in

agonist-induced internalization was shown to have different levels of expression in CHO, HEK293 and SH-SY cells (Santini et al., 2000).

Koenig and Edwardson (1994b) have studied the delivery of M_4 receptors in NG108 -15 cells, which express them. In their study, they used propyl benzilylcholine mustard to label the receptors remaining on the membrane after internalization caused by carbachol treatment (Koenig and Edwardson, 1994b). They devised a method for calculating receptor recycling by subtracting the plasma membrane delivery of M_4 receptors in unstimulated cells from the plasma membrane delivery of M_4 receptors in the cells stimulated with carbachol. Based on their data, they developed a mathematical model for the delivery of the receptors to the membrane incorporating the biosynthetic, endocytic, recycling and degradation compartments (Koenig and Edwardson, 1994a). They also found that the rate and extent of agonist-dependent internalization depends upon the muscarinic receptor subtype expressed in a cell line (Koenig and Edwardson, 1996). These observations provide further impetus to investigate the kinetics of M_1-M_5 receptor recycling and downregulation in the same cell line. Gill and Rang (1966) showed an aziridium ion derivative of benzilylcholine mustard (BCM) has a permanent antagonist activity with the muscarinic receptors. We used this derivative to alkylate receptors remaining on the plasma membrane, after internalization was initiated by carbachol in our recycling assays. BCM alkylates the muscarinic receptors at a greater rate than propyl benzilylcholine mustard allowing us to reduce the influence of alkylation on the rate of receptor recycling.

To date, the agonist-dependent internalization, recycling and downregulation of all five subtypes of muscarinic receptor have not been characterized in one cell type. This comparison is important because differences in the kinetics or extent of internalization, recycling or downregulation would be an indication that distinct mechanisms regulate the activity of muscarinic receptors in a subtype-specific manner. In this aim, the carbachol-dependent internalization and downregulation of M_1-M_5 receptors in CHO cells were compared. The recycling of M_1-M_5 receptors after a brief treatment with carbachol was also compared. Prior to

our study, the downregulation of M_4 receptors was not studied in CHO cells. The internalization of M_5 receptors was not studied in CHO cells and the agonist-induced downregulation and recycling of M_5 receptors was not characterized previously.

1.9.2 Characterization of the effect of proteasomal and lysosomal inhibitors on the downregulation of muscarinic receptors

To some extent, the mechanisms of agonist-induced internalization and desensitization of muscarinic receptors have been worked out. The mechanisms of agonist-induced downregulation of muscarinic receptors are unclear. Lysosomal inhibitors (e.g., NH_4Cl) have been previously shown to inhibit the agonist-induced downregulation of muscarinic receptors in NG-108 cells and pancreatic acini cells (Ray and Berman, 1989; Hootman et al., 1991) but they had no effect on the downregulation of muscarinic receptors in the vas deferens (Hiroshi et al., 1982). Lactacystin, a proteasomal inhibitor, inhibited the agonist-induced downregulation of M_2 receptors in MEF cells (Mosser et al., 2008). In MDCK cells, only a small fraction of M_1 receptors colocalize with the lysosomal marker Rab7 after agonist-stimulation, suggesting that there could be pathways alternate to the lysosomal pathway for the degradation of M_1 receptors (Shmuel et al., 2007). All these reports prompted us to think that there could be more than just the lysosomal pathway for agonist-stimulated degradation of muscarinic receptors. The mechanism of downregulation of muscarinic receptors has not been studied previously. Hence, this aim addresses whether the mechanism is lysosomal or proteasomal. In particular, the effect of proteasomal inhibitors and lysosomal inhibitors on the carbachol-induced downregulation of muscarinic receptor subtypes M_1-M_5 is characterized.

1.9.3 Characterization of domains in the third intracellular loop and C terminal tail of M_1 muscarinic receptors

The domains in the third intracellular loop and C terminal tail of GPCRs are involved in various agonist-dependent processes (internalization, recycling and downregulation). For example,

Hashimoto and Morisawa (2008) identified a sequence essential for internalization and recycling in the third intracellular loop of M_4 receptors. The recycling motif identified had no similarity with currently known recycling motifs for various GPCRs (Hashimoto et al., 2008). Further, the recycling sequence identified for M_4 receptors is present in the i3 loop while the recycling signals of other GPCRs are in the C-tail domain (Hanyaloglu and Zastrow, 2008; Hashimoto et al., 2008). Previously, our laboratory identified a di-cysteine motif in the i3 loop of M_1 receptors that is involved in agonist-induced internalization (Sawyer et al., 2008). Hence, we propose to characterize domains in the i3 loop and C-terminal tail of M_1 receptors to identify domains involved in agonist-dependent recycling and downregulation. To pursue this aim, two deletion mutants: i3 loop deletion mutant (M_1 del 276-282) and C-terminal tail deletion mutant (M_1 del 447-459) were made in M_1 receptors and the mutant receptors were stably expressed in CHO cells. Shown below is a snapshot of multiple sequence alignment of M_1-M_5 receptors in the proposed M_1 deletion regions.

```
M1              235 ----GGSSSS------SERSQPGAEGSPETPPGRCCRCCRAPRLLQAYSWKEEEEED--- 281
M5              263 QRERNQASWS------SSRRSTSTTGKPSQATGPSANWAKAEQLTTCSSYPSSEDEDKPA 316
M3              300 MKRSNRRKYGRCHFWFTTKSWKPSSEQMDQDHSSSDSWNNNDAAASLENSASSDEEDIGS 359
M2              238 ----GRIVKP------NNNNMP---SSDDGLEHNKIQNGKAPRDPVTENCVQGEEKESSN 284
M4              246 ----SPLMKQ------SVKKPPPGEAAREELRNGKLEEAPPPALPPPPRPVA--DKDTSN 294

M1              282 -EGSMESLTS---------SEGEE-PG---SEVVIKMPMVDPEAQAPTKQPP--RSSPNT 325
M5              317 TDPVLQVVYK---------SQGKESPGEEFSAEETEETFVKAETEKSDYDTPNYLLSPAA 367
M3              360 ETRAIYSIVLKLPGHSTILNSTKLPSSDNLQVPEEELGMVDLERKADKLQAQKSVDDGGS 419
M2              285 DSTSVSAVAS----------NMR--DDEITQDENTVSTSLG-----HSKDENSKQTCIRI 327
M4              295 ESSSGSATQN----------TKERPATELSTTEATTPAMPAPPLQPRALNPASRWSKIQI 344

M1              411 STINPMCYALCNKAFRDTFRLLLLCRWDKRRWRKIPKRPGS----VHRTPSRQC 460
M5              488 STVNPICYALCNRTFRKTFKMLLLCRWKKKKVEEKLYWQGN----SKLP----- 532
M3              537 STVNPVCYALCNKTFRTTFKMLLLCQCDKKKRRKQQYQQRQSVIFHKRAPEQAL 590
M2              433 STINPACYALCNATFKKTFKHLLMCHYKNIGATR-------------------- 466
M4              446 STINPACYALCNATFKKTFRHLLLCQYRNIGTAR-------------------- 479
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The binding and signaling parameters of the mutant receptors stably expressed in CHO cells were characterized. Additionally, the kinetics and extent of carbachol-induced internalization, recycling and downregulation of the mutant receptors were also compared with wild-type M_1 receptors.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Proteasome inhibitor I (PSI), E-64-d, Lactacystin and MG-132 were purchased from Enzo lifesciences (Farmingdale, NY). Ammonium chloride and atropine was purchased from Sigma-Aldrich (St Louis, MO). [^{3}H]NMS ([^{3}H]-N-methylscopolamine, specific activity 70 Ci/mmol) and [^{3}H]QNB ([^{3}H]quinuclidinyl benzylate, specific activity 50.5 Ci/mmol) were purchased from Perkin Elmer lifesciences (Boston, MA). Scintiverse BD cocktail was purchased from Fisher Scientific (Fairlawn, NJ). BCA protein assay kit was purchased from Pierce (Rockford, IL). Lipofectamine 2000 and geneticin were purchased from Invitrogen (Carlsbad, CA). Quikchange II site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). M_1 receptor cDNA in a modified Okayama-Berg expression vector (pCD) and CHO cells stably expressing muscarinic receptors (M_1-M_5) individually were generous gifts from Dr. Tom I. Bonner at the National Institute of Mental Health, Bethesda, MD. Benzilylcholine mustard (BCM) was a kind gift of Dr. Fred Ehlert at University of California, Irvine, CA. Complete Mini EDTA-free protease inhibitor cocktail tablet was purchased from Roche diagnostics (Indianapolis, IN). GF/B-fired glass-fiber filters were purchased from Whatman (Clifton, NJ).

2.2 Cloning and site-directed mutagenesis

Deletion mutations were created using carefully designed mutagenesis primers following

Quikchange II site directed mutagenesis protocol. M_1 del 276-282 DNA in pCD vector was created by amplifying M_1 wild-type DNA in pCD vector using Quikchange II XL site directed mutagenesis kit with mutagenesis primers 5' CCTACAGCTGGAAGGGCTCCATGGAGTCC 3' and 5'GGACTCCATGGAGCCCTTCCAGCTGTAGG 3'. After mutagenesis, M_1 del 276-282 DNA was amplified using primers 5' GCAGAGGAATTCGTCATGAACACTTCAGCCCCAC 3' and 5' GCAGAGGCGGCCGCTCAGCATTGGCGGGAG 3' to generate M_1 del 276-282 DNA with Eco RI and Not I restriction sequences at the ends. For subcloning into pIRES neo vector containing mammalian selection marker aminoglycoside 3'-phosphotransferase in preparation for creation of stable cells, both pIRES neo vector and the PCR product was digested with Eco RI and Not I enzymes and ligated using T4 DNA ligase.

Similarly, M_1 del 447-459 DNA in pCD vector was created by amplifying M_1 wild-type DNA in pCD vector using mutagenesis primers 5' GCTGGCGCAAGATCCCCTGCTGATAGTCC 3' and 5' GGACTATCAGCAGGGGATCTTGCGCCAGC 3'. Following mutagenesis, M_1 del 447-459 DNA was selectively amplified using primers 5' GCAGAGGAATTCGTCATGAACACTTCAGCCCCAC 3' and 5' GCAGAGGCGGCCGCTCAGCATTGGCGGGAG 3'so that the resultant PCR product has Eco RI and Not I restriction enzyme sequences at its ends. For subcloning into pIRES neo vector, both pIRES neo vector and the PCR product was digested with Eco RI and Not I enzymes and ligated using T4 DNA ligase enzyme. All the plasmids generated using mutagenesis or subcloning were sequenced at OSU Stillwater core DNA sequencing facility and verified.

2.3 Cell culture

CHO cells with a low passage number were plated in a 35 mm cell culture dish at a cell density of 3.3×10^5 cells per ml of F-12K media supplemented with 10% FBS. The following day M_1 del 276-282 or M_1 del 447-459 DNA in pIRESneo vector was transfected using Lipofectamine 2000 following the manufacturer's protocol. The transfected cells were trypsinized and passed

into 10 cm cell culture dishes at a ratio of 1 in 1000 or less on the third day. A concentration of 500 µg of geneticin (Invitrogen, CA) per ml in growth medium (F-12K, supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin) was used as selection pressure to isolate cells that took up the plasmid. The growth media in the dishes was exchanged with new media containing geneticin (500 µg/ml) once in every 3 days, for ten days. Single discrete colonies were selected at the end of ten day period by placing clonal rings on top of the colonies. Cells from various colonies were trypsinized and transferred into individual wells of 24-well plates containing 500 µl of media and geneticin. The level of expression of the mutant receptors in CHO cells was determined by performing an intact whole cell [^{3}H]NMS binding assay (see section 2.5 "*Receptor binding assays*"). Three colonies with the highest receptor expression as evident from the binding assay data were grown in T25 flasks and then stepped up into a T75 flask. After attaining confluence in a T75 flask, the stable cells were frozen in cryo vials with 5% DMSO in the vapor phase of liquid nitrogen and were revived in growth medium.

CHO cells stably expressing one of the subtypes of muscarinic receptors (M_1-M_5), M_1 del 276-282 or M_1 del 447-459 were passed a minimum of three times before use in experiments and were not passed more than twenty times. For experiments, CHO cells stably expressing individual muscarinic receptors (M_1-M_5), M_1 del 276-282 or M_1 del 447-459 were plated into 24-well plates at a density of 1.65 x 10^5 cells per well in growth medium (500 µl). Cells were then maintained in a humidified incubator set at 37 ^{o}C in an atmosphere of 5% CO_2 /95% air for 24 h, at which time the medium in each well of each plate was exchanged for fresh growth medium (500 µl). The cells were then maintained in the humidified incubator for an additional 24 h (48 h total) before conducting experiments.

2.4 Receptor trafficking assays

2.4.1 Receptor internalization assay

CHO cells stably expressing one of the subtypes of muscarinic receptor or mutant M_1 receptors were plated in 24-well plates as described above in section 2.3 "*Cell culture*". Cells

were washed thrice with F-12K to remove serum and then incubated with F-12K containing saturating concentration of muscarinic agonist carbachol (1 mM) for different time periods (6 wells for each time point) in a humidified incubator set at 37 ^{o}C in an atmosphere of 5% CO_2/95% air. Carbachol-induced internalization of muscarinic receptor subtypes or mutant M_1 receptors plateaued or nearly plateaued after 4 h of treatment with carbachol (1 mM). Hence, the time periods (0 to 4 h) before plateau is reached is used in the internalization experiments. Following carbachol treatment, cells were washed extensively on ice thrice with ice-cold PBS. Intact, whole cell binding assays were then performed using a single concentration (1.6 nM) of the membrane impermeable muscarinic receptor selective radioligand [^{3}H]*N*-metylscopolamine as described below in section 2.5 "*Receptor binding assays*". Specific [^{3}H]NMS binding is used to determine the amount of receptor expressed on the cell surface.

2.4.2 Receptor recycling assay

CHO cells stably expressing one of the subtypes of muscarinic receptor or mutant M_1 receptors were plated in 24-well plates (60 wells) as described above in section 2.3 "*Cell culture*". On the day of the experiment, plates were divided into two equal groups (30 wells per group) and cells were washed thrice with F-12K (prewarmed to 37 ^{o}C) to remove serum. Following the wash with F-12K, the first group of cells was incubated with F-12K (prewarmed to 37 ^{o}C) for 1 h in a humidified incubator set at 37 ^{o}C in an atmosphere of 5% CO_2/95% air. The first group of cells was used to determine the amount of receptor delivered to the plasma membrane in a constitutive manner. At the same time, the second group of cells was incubated with F-12K medium containing 1 mM carbachol for 1 h in a humidified incubator set at 37 ^{o}C. This incubation time was chosen because it was the minimal time necessary to obtain maximal or nearly maximal receptor internalization for all five subtypes of receptor and mutant M_1 receptors. The second group of cells was used to measure the amount of constitutive receptor plasma membrane delivery plus recycled receptor. After the 1 h incubation period, both groups of cells were washed thrice with F-12K (3 x 500 µl) on ice. This wash was conducted on ice to halt receptor

trafficking. Cells from both groups were incubated with cyclized BCM (50 nM) for 5 min in a humidified incubator set at 37 ^{o}C. BCM (10 µM) was cyclized in PBS during a 30 min incubation at 37 ^{o}C. The half-time for BCM cyclization is 2.5 min at 37 ^{o}C, thus a 30 min incubation is adequate to ensure complete cyclization of BCM to its membrane-impermeable aziridinium form (Gill and Rang, 1966). Cells were washed on ice with F-12K (3 x 500 µl) to remove BCM and then incubated for various periods of time for up to 90 min (6 wells for each time point in untreated and carbachol-treated plates) in a humidified incubator set at 37 ^{o}C. Cells were then washed with ice-cold PBS (3 x 500 µl) and the amount of receptor expressed on the plasma membrane was determined using intact, whole cell [^{3}H]NMS binding assays as described below in section 2.5 "*Receptor binding assays*".

As a control, 12 wells of a 24-well plate were plated at the same time as experimental plates. Each well of the control plate was washed with F-12K (3 x 500 µl) to remove serum and then incubated in the absence (6 wells) or presence of carbachol (1 mM; 6 wells) for 1 h in an incubator set at 37 ^{o}C in an atmosphere of 5% CO_2/95% air. Each well was washed with ice-cold PBS (3 x 500 µl) and then used in intact, whole cell [^{3}H]NMS binding assays. The specific [^{3}H]NMS binding of untreated wells was used to normalize specific binding of experimental plates. The specific [^{3}H]NMS binding of carbachol treated wells was used to determine how much internalization occurred during the 1 h treatment with carbachol.

2.4.3 Receptor downregulation assay

CHO cells stably expressing one of the subtypes of muscarinic receptor or mutant M_1 receptors were plated in a 24-well plate as described above under section 2.3 "*Cell culture*". Plates were divided into two equal groups (12 wells per group) and cells were washed with F-12K (3 x 500 µl) to remove serum. The first group of cells was used as a control. This group of cells was incubated with F-12K (preheated to 37 ^{o}C) for 24 h in a humidified incubator set at 37 °C and in an atmosphere of 5% CO_2/95% air. The second group of cells was incubated with F-12K medium containing 1 mM carbachol for 24 h to induce receptor downregulation. This incubation

time was chosen because muscarinic receptor downregulation is a slow process that requires approximately 24 h for a substantial loss of receptor to be observed (Shockley et al., 1997). Cells from both groups were washed extensively on ice with ice-cold PBS (3 x 500 µl). Intact, whole cell binding assays were then performed using a single concentration of [^{3}H]NMS (6 wells in untreated and carbachol treated plates) to determine the amount of plasma membrane expressed receptor. To determine the total amount of muscarinic receptor in CHO cells, intact, whole cell binding assays using a single concentration of [^{3}H]QNB (6 wells in untreated and carbachol treated plates) were also performed as described below in section 2.5 "*Receptor binding assays*."

2.4.4 Downregulation assay in the presence of inhibitors

CHO cells stably expressing each subtype of muscarinic receptor were plated into 24-well plates as described above in section 2.3 "*Cell culture*". On the day of the experiment, cells were divided into two equal groups: control and carbachol-treatment groups. The control group was treated with vehicle, proteasomal inhibitors (PSI, MG-132, lactacystin) or lysosomal inhibitors (NH_4Cl, E-64-d) for 24.5 h. The carbachol treatment group was treated with vehicle, proteasomal or lysosomal inhibitors for 30 minutes, following which carbachol (1 mM) is added and the cells were allowed to incubate with carbachol in the continued presence of vehicle, proteasomal or lysosomal inhibitors for 24 hours. Cells from both groups were washed extensively on ice with ice-cold PBS (3 x 500 µl) and then used in intact, whole cell binding assays using a single concentration of the membrane permeable radioligand [^{3}H]QNB (1.2 nM) (see section 2.5 "*Receptor binding assays*"). To get an estimate of receptor downregulation, specific [^{3}H]QNB binding in cells treated with carbachol in the presence of inhibitor/vehicle was divided by the specific binding obtained in cells treated with the inhibitor/vehicle alone, and subtracted from 100. The significance of differences between the effects of these inhibitors on carbachol-induced downregulation was determined using a one-way ANOVA and Dunnett's post hoc test (GraphPad Prism, ver. 5.01; San Diego, CA).

2.4.5 Time-course downregulation assay

CHO cells expressing each muscarinic receptor subtype were plated into 24-well plates as described above in section 2.3 "*Cell culture*". Cells were washed three times with 500 µl of F-12K medium to remove serum and then incubated with carbachol (1 mM) in F-12K medium for various time intervals up to 24 hours in a humidified CO_2 incubator. Cells were washed extensively to remove carbachol (3 x 500 µl PBS) and then used in intact, whole cell [^{3}H]QNB binding assays as described below in section 2.5 "*Receptor binding assays*".

2.4.6 Assay to determine the potency of carbachol-induced downregulation

CHO cells expressing a subtype of muscarinic receptor were plated into 24-well plates as described above in section 2.3 "*Cell culture*". Cells were then treated with equally spaced concentrations of carbachol for 7 or 24 hours. Cells were washed extensively to remove carbachol (3 washes x 500 µl PBS) and then used in intact, whole cell [^{3}H]QNB binding assays as described below in section 2.5 "*Receptor binding assays*". The concentration of carbachol eliciting half-maximal response (downregulation) EC_{50} was estimated using non-linear regression analysis (GraphPad Prism, ver. 5.01; San Diego, CA).

2.5 Receptor binding assays

Intact, whole cell binding assays were performed using either a single concentration of [^{3}H]NMS (1.6 nM) or [^{3}H]QNB (1.2 nM). The plasma membrane expressed muscarinic receptor was quantified using [^{3}H]NMS and the total muscarinic receptor expressed was measured using [^{3}H]QNB. 1.6 nM [^{3}H]NMS should occupy approximately 86%, 72%, 84%, 94% and 60% of human M_1, M_2, M_3, M_4 and M_5 receptors, respectively (Ehlert et al., 1996) while 1.2 nM [^{3}H]QNB should occupy approximately 97%, 98%, 93%, 97% and 96% of human muscarinic M_1, M_2, M_3, M_4 and M_5 receptors, respectively (Bolden et al., 1992). A control was used in each assay (i.e., internalization, downregulation and recycling) to determine specific [^{3}H]NMS or [^{3}H]QNB binding in untreated CHO cells expressing a particular receptor subtype. All other binding data from each assay was divided by the control binding and then reported as percent of control.

Subtype-specific differences in affinity for [^{3}H]NMS and [^{3}H]QNB were accounted for by this transformation.

Washed cells were incubated with either [^{3}H]NMS or [^{3}H]QNB in the absence (three wells for each time point; total binding) and presence (three wells for each time point; nonspecific binding) of atropine (10 µM) in 500 µl binding buffer (25 mM HEPES, 113 mM NaCl, 6 mM dextrose, 3 mM $CaCl_2$, 3 mM KCl, 2 mM $MgSO_4$, 1 mM NaH_2PO_4, pH 7.4) for either one h ([^{3}H]NMS) or 18 h ([^{3}H]QNB) at 4^{o}C. Unbound [^{3}H]NMS or [^{3}H]QNB was then removed by rapidly and gently washing cells with ice-cold PBS (2 x 1 ml). The cells were solubilized with 500 µl of NaOH (0.25 N) and neutralized with 70 µl of HCl (2.5 N) and pipetted into scintillation vials (Griffin et al., 2003). 5 ml of scintiverse BD cocktail was added to the vials and counted using a Beckman LS 6500 scintillation counter.

BCA protein assay was performed to determine the average amount of protein expressed in CHO cells for each radioligand binding assay performed. Three wells of a 24-well plate were plated at the same time as experimental plates as described in section 2.3 "*Cell culture*". Cells were treated the same as the cells in each experiment performed and then cells were washed two times with 500 µl mannitol wash buffer (0.29 M mannitol, 0.01 M Tris, 0.5 mM $Ca(NO_3)_2$, pH 7.4) (Goldschmidt and Kimelberg, 1989). The protein concentration was determined for each well using the bicinchoninic acid (BCA) protein assay kit following manufacturer's protocol.

2.5.1 Filtration receptor binding assays

To verify if BCM irreversibly binds muscarinic receptors, CHO M_5 cells were plated (5 x 10^6 cells/plate) in six 10-cm dishes in growth medium (15 ml, see section 2.3 "*Cell culture*"). The very next day, cells were washed three times with F-12K (15 ml) to remove serum. Five of the six plates were incubated with cyclized BCM (50 nM) in F-12K (15 ml) for 5 min at 37 ^{o}C. The sixth plate was incubated in F-12K for 5 min at 37 ^{o}C. Following the incubation period, cells on each plate were rapidly washed (3 times, 15 ml) with ice-cold PBS to remove unbound BCM. Cells were then incubated for 5 min at room temperature with 5 ml hypotonic buffer (1 mM Tris-HCl, 1

mM EGTA, 1 mM $MgCl_2$, 120 mM sucrose, pH 7.6) (Chang et al., 1981). Cells were scraped from plates and transferred into individual tubes (15 ml conical), then homogenized using a Tissue-Tearor™ on setting five (15 s). The untreated homogenate was retained on ice. The BCM treated homogenates were incubated in a humidified incubator set at 37 °C, 5% CO_2/95% air for 0, 15, 30, 60 or 90 min, then retained on ice. Aliquots of homogenates (100 µl) were then incubated in 12 x 75 mm polypropylene tubes with [^{3}H]NMS (1.6 nM) in binding buffer (see section 2.5 "*Receptor binding assays*", 400 µl) in the absence (3 tubes, total binding) and presence (3 tubes, nonspecific binding) of atropine (10 µM) for 1 h at 4 °C with constant shaking. Bound [^{3}H]NMS was trapped on Whatman glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD). The filters were washed three times with approximately 3 ml of ice-cold saline (0.9%) each wash. The assay was repeated twice.

To verify that [^{3}H]QNB permeates intracellular compartments containing muscarinic receptor in intact, whole CHO cells, CHO M_1 cells were plated (5 x 10^6 cells/plate) in two 10-cm dishes in growth medium (15 ml, see Section 2.3 "*Cell culture*"). The very next day, cells were washed three times with sterile F-12K (15 ml) to remove serum. Plates were then incubated for 24 h in a humidified incubator set at 37 °C in an atmosphere of 5% CO_2/95% air in the absence (one plate) and presence (one plate) of carbachol (1 mM). Cells on each plate were washed (3 times, 15 ml) with ice-cold PBS to remove carbachol and then incubated for 5 min at room temperature with hypotonic buffer (5 ml). Following the incubation in hypotonic buffer, cells were scraped from plates and transferred into individual tubes (15 ml conical), then homogenized using a Tissue-Tearor™ on setting five (15 s). The homogenates were pelleted (100,000 x g, 1 h) and resuspended in 1 ml binding buffer (see section 2.5 "*Receptor binding assays*") containing protease inhibitor. Aliquots of homogenates (100 µl) were then incubated in 12 x 75 mm polypropylene tubes with [^{3}H]QNB (1.6 nM) in binding buffer (400 µl) in the absence (3 tubes, total binding) and presence (3 tubes, nonspecific binding) of atropine (10 µM) for 6 h at 4 °C with constant shaking. Bound [^{3}H]QNB was trapped on Whatman glass-fiber filters using a cell

harvester (Brandel, Gaithersburg, MD). The filters were washed three times with approximately 3 ml of ice-cold saline (0.9%) each wash. The assay was repeated twice.

2.5.2 Saturation binding assays

Saturation binding assay was conducted on CHO cells stably expressing M_1 del 276-282 or M_1 del 447-459 receptors to estimate the equilibrium dissociation constant (K_D) of the radioligand [^{3}H]NMS for the mutant receptors. CHO cells stably expressing the mutant M_1 receptors were plated at a density of 1.65 x 10^5 cells/well. The following day, the media in the wells was exchanged with fresh F-12K media. The very next day, the cells were washed three times with ice-cold PBS to remove serum. 400 µl of binding buffer was added to non-specific wells while 450 µl of binding buffer was added to the total wells. 50 µl of 10 µM atropine was added to the non-specific wells. Then 50 µl of geographically spaced concentrations of the [^{3}H]NMS was added to both the total and nonspecific wells and incubated at 4 ^{o}C for 2 hours to equilibrate. The cells were then washed twice with 1 ml PBS to get rid of unbound ligands and solubilized by incubation with 0.25N NaOH for 30 minutes. 70 µl of 2.5N HCl was added to neutralize the solubilized cells containing NaOH. The neutralized extracts were transferred into scintillation vials. 5 ml of scintiverse was added to the scintillation vials and radioactivity was counted using a Beckman LS 6500 scintillation counter.

2.6 Phosphoinositide hydrolysis assays

Phosphoinositide hydrolysis assays were conducted on CHO cells stably expressing the wild-type or mutant M_1 receptors as described previously (Sawyer et al., 2006). CHO cells stably expressing the wild-type or mutant M_1 receptors were washed (3 x 500 µl) with F-12K medium to remove serum and then incubated with 0.2 µM [^{3}H]myo-inositol (PerkinElmer, Boston, MA) in F-12K (500 µl) for 18 h in a humidified incubator. The cells were washed the following day with F-12K (2 x 500 µl) to remove unincorporated inositol with 10 min incubation in a humidified incubator between each wash. On the third wash, cells were washed with F-12K (270 µl) containing LiCl (10 mM). The washed cells were then incubated with F-12K (300 µl) containing

LiCl (10 mM) and geometrically spaced concentrations of carbachol (0.5 log unit) for 30 min in a humidified incubator at 37 ^{o}C in an atmosphere of 5% CO_2/95% air. The medium was aspirated after the 30 minute incubation period. The cells were incubated on ice for 15 min with ice-cold 5% perchloroacetic acid PCA (200 µl). 0.525 M KOH in 10 mM Tris HCl (360 µl) was added to each well and the cells were incubated for an additional 15 min on ice. The neutralized extracts (approximately 565 µl) were transferred immediately into 1.5-ml microcentrifuge tubes and each well was washed with 25 mM Tris HCl (400 µl), pH 7.4. Each wash was added to the corresponding microcentrifuge tube. The tubes were centrifuged at 3000 x g for 10 min to pellet cell debris. The supernatant was transferred to glass tubes containing 25 mM Tris HCl (2 ml), pH 7.4 and mixed. The mixture was then applied to individual 1 ml Dowex AG 1-X8 (HCL form, 100-200 mesh) columns. Columns were washed three times with 2 ml water and [^{3}H]inositol phosphates were eluted after washing using 1 M hydrochloric acid (2.5 ml) into 25-ml scintillation vials. 20 ml of scintillation cocktail was added and counted using a Beckman LS 6500 scintillation counter.

2.7 Data Analysis

Significance values (*P* values) calculated using either Student's t-test, one-way ANOVA with Tukey or Dunnett's post-hoc test (GraphPad Prism, ver. 5.01; San Diego, CA), are reported where appropriate (GraphPad Prism, ver. 5.01; San Diego, CA). Data with a *P* value < 0.05 was considered significant. Estimates of the rate constants for muscarinic M_1-M_5 receptors or mutant M_1 receptors internalization were made by fitting data using a single-phase exponential decay equation (GraphPad Prism, ver. 5.01; San Diego, CA). Estimates of the rate constants for the plasma membrane delivery of muscarinic M_1-M_5 receptors or mutant M_1 receptors in untreated and carbachol treated cells were made by fitting data using a single-phase exponential association equation (GraphPad Prism, Ver. 5.01; San Diego, CA).

CHAPTER III

RESULTS

3.1 Characterization of internalization, recycling and downregulation of muscarinic receptors

3.1.1 Comparison of M_1-M_5 receptor internalization

We investigated the kinetics and extent of agonist-induced internalization of human muscarinic M_1-M_5 receptors. CHO cells expressing M_1-M_5 receptors were incubated with the muscarinic receptor selective agonist carbachol (1 mM) for various times up to four hours and then receptor binding at the cell surface was measured using a single concentration of [^{3}H]NMS (1.6 nM). As shown in Figure 1, the internalization of all five subtypes, as determined by decreased specific [^{3}H]NMS binding, was consistent with a first-order decay process.

The rate constants for carbachol-induced internalization of each subtype over the time interval of 0 to 4 h are estimated and tabulated (Table 1). M_2 receptors internalized quickly and to a greater extent, when compared to M_1, M_3, M_4 and M_5 receptors. The half-time ($t_{½}$) for M_2 receptor internalization was 8.6 min and specific [^{3}H]NMS binding decreased 87% during the 4 h treatment with carbachol (Figure 1B and Table 1). The half-times for the carbachol-induced internalization of M_1 and M_3 receptors were similar ($t_{1/2}$ = 46.3 min and 35.5 min, respectively), but were significantly longer than that for M_2 receptors (Figures 1A and C, Table 1). Specific [^{3}H]NMS binding decreased 45% and 41% in CHO cells expressing M_1 and M_3 receptors,

respectively, during carbachol treatment (Figures 1A and C, Table 1). The half-times for M_4 and M_5 receptor internalization were also similar ($t_{1/2}$ = 12.8 min and 13.1 min, respectively) and significantly longer than that for M_2 receptors (Figures 1D and E, Table 1). During the 4 h carbachol treatment, specific [^{3}H]NMS binding decreased 75% in CHO cells expressing M_4 receptors and 65% in CHO cells expressing M_5 receptors (Figures 1D and E, Table 1). Overall, we observed subtype-specific differences in the rates and extent of internalization between the subtypes of muscarinic receptors.

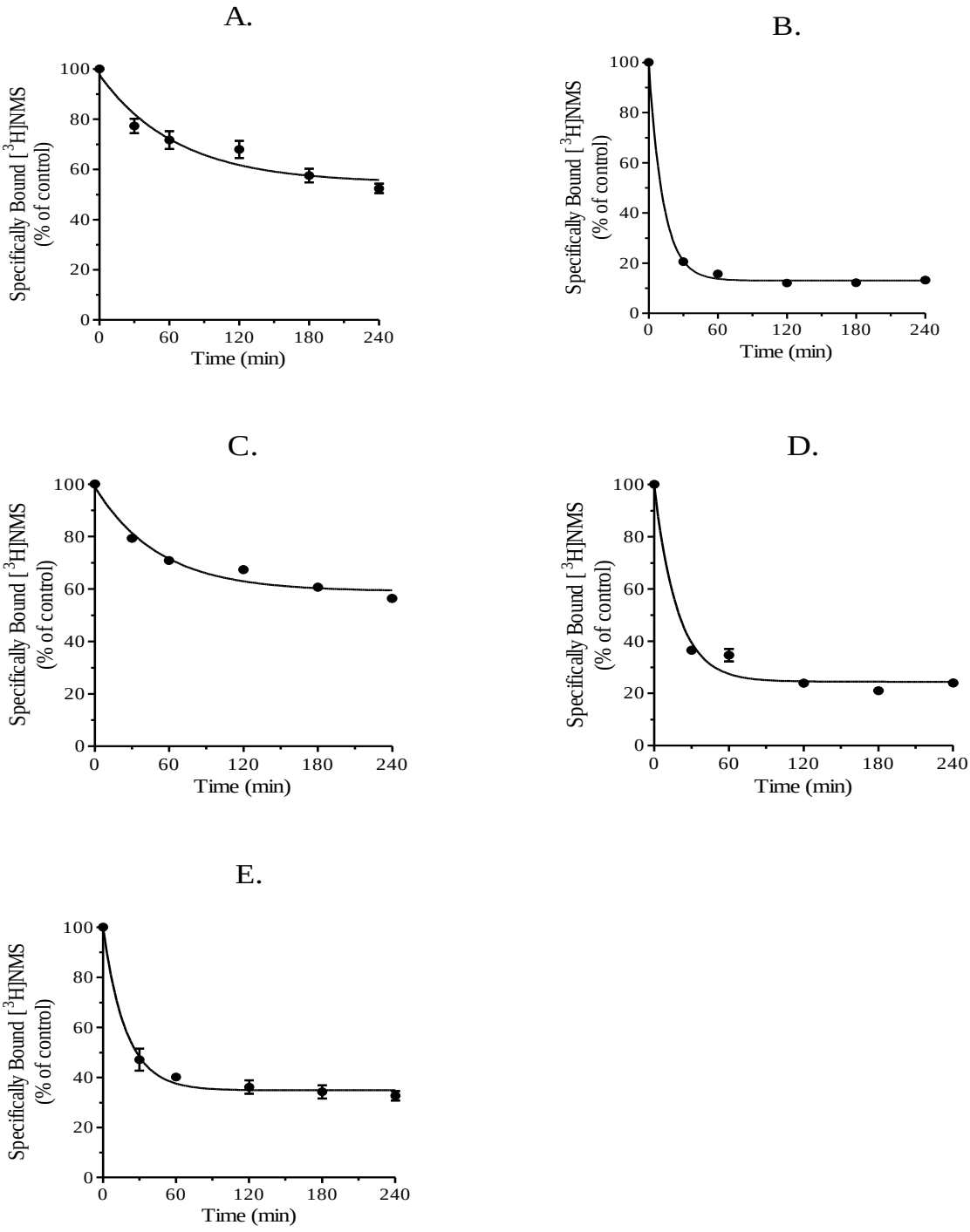

Figure 1. Internalization of M_1-M_5 receptors. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D) or M_5 (E) receptors were incubated with carbachol (1 mM) for various periods of time for up to 240 min at 37 ^{o}C. Cells were then used in intact, whole cell [^{3}H]NMS binding assays as described in section 2.5 "*Receptor binding assays*". Each data point represents the mean ± S.E.M. of three experiments conducted in triplicate.

Table 1: Comparison of specific [^{3}H]NMS binding and rate constants for M_1-M_5 receptor internalization[a].

Muscarinic receptor subtype[b]	Specific [^{3}H]NMS binding (fmol/mg protein)		K (min^{-1})[d,e]	Plateau (% of control)[f,g]
	Control	Carbachol treated[c]		
M_1 (3)	1395.2 ± 42.5	731.5 ± 34.1	0.015 ± 0.004[h]	54.6 ± 3.4[i]
M_2 (3)	177.6 ± 12.8	23.8 ± 3.0	0.080 ± 0.005[j]	13.0 ± 0.53[k]
M_3 (3)	549.4 ± 42.7	309.3 ± 21.2	0.020 ± 0.002[l]	59.1 ± 1.4[m]
M_4 (3)	439.2 ± 16.3	105.5 ± 5.5	0.054 ± 0.006	24.5 ± 1.6[n]
M_5 (3)	262.6 ± 31.6	84.6 ± 5.0	0.053 ± 0.007	34.8 ± 1.4

[a] Data from Figure 1.

[b] Number of experiments is shown in parenthesis.

[c] Binding observed after 4 h of carbachol treatment.

[d] The rate constant for internalization was determined by fitting data shown in Figure 1 to a single-phase decay equation (see Section 2.7 "*Data analysis*").

[e] The rate constants for carbachol-induced internalization differed significantly across the receptor subtypes ($p < 0.0001$) as determined by one-way ANOVA.

[f] The plateau for internalization was determined by fitting data shown in Figure 1 to a single-phase decay equation (see Section 2.7 "*Data analysis*").

[g] The plateaus for carbachol-induced internalization differed significantly across the receptor subtypes ($p < 0.0001$) as determined by one-way ANOVA.

[h] Tukey post-hoc comparisons indicate that the rate constant (K) of M_1 receptors is significantly different ($p < 0.01$) from M_2, M_4, and M_5 receptors.

[i] Tukey post-hoc comparisons indicate that the plateau of M_1 receptors is significantly different from M_2 ($p < 0.001$), M_3 ($p < 0.05$), M_4 ($p < 0.001$), and M_5 ($p < 0.001$) receptors.

[j] Tukey post-hoc comparisons indicate that the rate constant (K) of M_2 receptors is significantly different ($p < 0.01$) from M_3, M_4, and M_5 receptors.

[k] Tukey post-hoc comparisons indicate that the plateau of M_2 receptors is significantly different ($p < 0.001$) from M_3, M_4, and M_5 receptors.

[l] Tukey post-hoc comparisons indicate that the rate constant (K) of M_3 receptors is significantly different ($p < 0.01$) from M_4 and M_5 receptors.

[m] Tukey post-hoc comparisons indicate that the plateau of M_3 receptors is significantly different ($p < 0.001$) from M_4, and M_5 receptors.

[n] Tukey post-hoc comparisons indicate that the plateau of M_4 receptors is significantly different ($p < 0.001$) from M_5 receptors.

3.1.2 Comparison of recycling of M_1-M_5 receptors

The assay for receptor recycling in CHO cells consisted of three sequential phases: 1) incubation of cells with carbachol (1 mM, 1 hr) followed by washing, 2) treatment with BCM followed by washing, and 3) measurement of the binding of [^{3}H]NMS (1.6 nM) at various times up to 90 min. An increase in phase 3 binding in response to phase 1 carbachol treatment was defined as recycling. Control cells received no carbachol during phase 1.

BCM is an irreversible muscarinic receptor selective antagonist that alkylates M_1-M_5 receptors. To ensure selective alkylation of plasma membrane expressed receptor, BCM was cyclized at 37 ^{o}C for 30 min, which results in a 99.99% conversion of the parent mustard to a membrane impermeable quaternary aziridinium ion (Gill and Rang, 1966). CHO cells stably expressing M_3 receptors were also incubated with increasing concentrations of cyclized BCM (10 nM, 50 nM, and 100 nM) for either 5, 10 or 20 min (Figure 2 and 3). From these data, it was determined that a 5 min treatment with 50 nM BCM was adequate to alkylate approximately 95% of plasma membrane expressed M_3 receptor (Figure 3). Since a majority of M_3 receptors were alkylated using these conditions, CHO cells expressing any of the muscarinic receptors were incubated at 37 ^{o}C for 5 min with BCM (50 nM) in phase 2 of recycling experiments. Additionally, this short treatment with BCM should minimize the amount of receptor trafficking to the plasma membrane during the alkylation phase (phase 2) of recycling experiments.

Intact, whole cell [^{3}H]NMS binding assays (see section 2.5 "*Receptor binding assays*") were performed on untreated and BCM treated CHO cells expressing M_1-M_5 receptors. When compared to untreated cells, specific [^{3}H]NMS binding in BCM treated cells (5 min, 50 nM) expressing M_1, M_2, M_3, M_4 and M_5 receptors decreased 90.4 ± 1.8%, 93.9 ± 1.0%, 97.0 ± 0.3%, 94.3 ± 0.7% and 85.4 ± 2.7%, respectively (Figure 3).

From our preliminary studies, we found that M_5 receptors had the greatest extent of recycling (Figure 4E and Table 2). Hence, we performed filtration binding assays (see section 2.5.1 "*Filtration receptor binding assays*") on homogenates of untreated and BCM treated CHO

cells expressing M_5 receptors to prove that BCM irreversibly alkylates muscarinic receptors. Intact, whole CHO M_5 cells were incubated at 37 ^{o}C for 5 min in the absence (control) and presence of cyclized BCM (50 nM). Cells were washed and homogenized and the untreated homogenate was retained on ice. The BCM treated homogenates were incubated for 0, 15, 30, 60 and 90 min at 37 ^{o}C. 5 min BCM treatment caused a 82% reduction in specific [^{3}H]NMS binding and a similar reduction was observed after 15 (83%), 30 (82%), 60 (84%) and 90 (85%) min incubations at 37 ^{o}C (data not shown). These data indicate that BCM irreversibly alkylates the plasma membrane expressed M_5 receptor under conditions similar to those used in recycling assays. Presumably, BCM irreversibly alkylates M_1-M_4 receptors in recycling assays as well.

In CHO cells expressing the muscarinic M_2 receptor, 1 h carbachol treatment (1 mM) caused a 79% reduction in specific [^{3}H]NMS binding (Table 2). After BCM alkylation (50 nM, 5 min), the initial rate of recovery of [^{3}H]NMS binding in CHO cells expressing the M_2 receptor was comparable in untreated and carbachol-treated cells, indicating no significant recycling (Figure 4B and Table 2).

Unlike the M_2 receptor, M_1, M_3, M_4, and M_5 receptors recycled after carbachol-induced internalization, albeit at different rates and to different extents. When expressed relative to control, the initial rate of recycling was 2.5-, 3.8-, 3.1- and 8.9-fold greater in carbachol treated cells expressing M_1, M_3, M_4 and M_5 receptors, respectively. The corresponding maximal increases in binding at 90 min were 1.4-, 1.6-, 1.4- and 3.5-fold respectively. These data are summarized in Table 2 and Figures 4A, C-E.

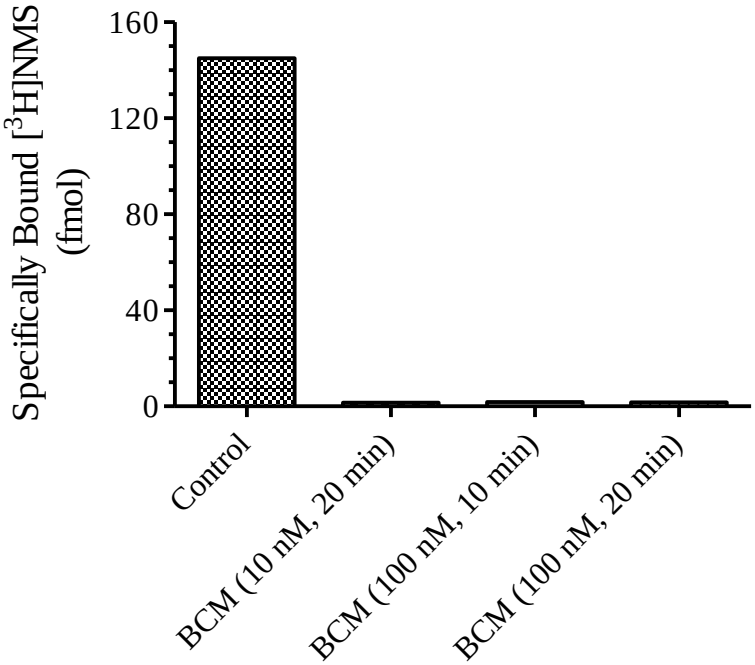

Figure 2. BCM alkylation of M_3 receptors. CHO cells stably expressing M_3 receptors were treated with either 10 nM or 100 nM cyclized BCM for 10 or 20 minutes. After extensive washing, intact whole cell binding assays using [^{3}H]NMS (1.6 nM) were performed as described in section 2.5 "*Receptor binding assays*". Each bar represents a single experiment conducted in triplicate.

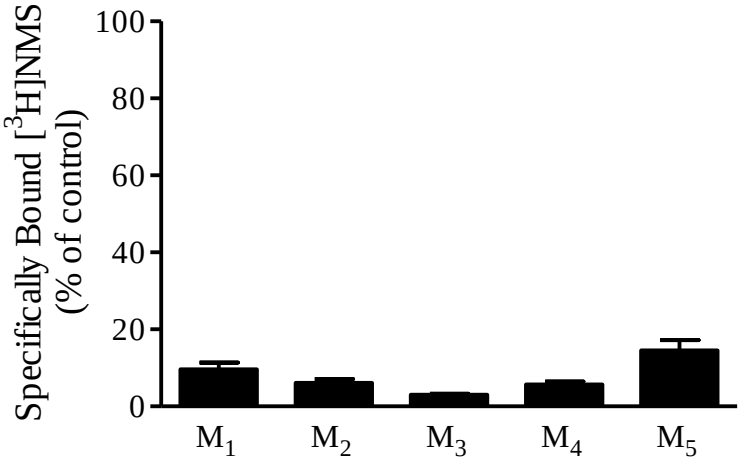

Figure 3. BCM alkylation of M_1-M_5 receptors. CHO cells stably expressing either muscarinic M_1, M_2, M_3, M_4 or M_5 receptors were treated with cyclized (see section 2.4.2 "*Receptor recycling assay*") BCM (50 nM) for 5 min at 37^{o}C. After extensive washing, intact whole cell [^{3}H]NMS binding assays were performed as described in section 2.5 "*Receptor binding assays*". Each bar represents the mean ± S.E.M. of three to four experiments conducted in triplicate.

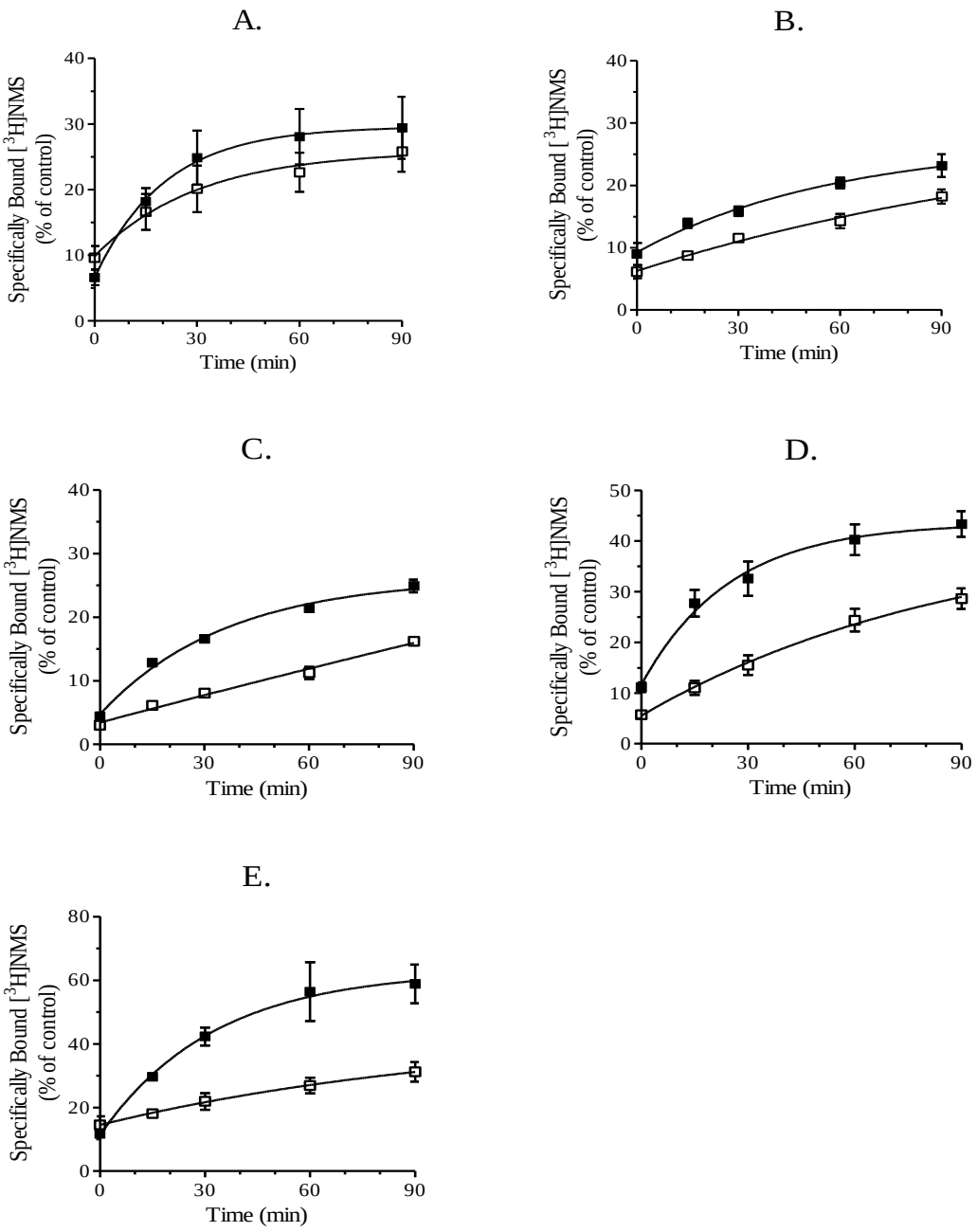

Figure 4. Recycling of M_1-M_5 receptors. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D) or M_5 (E) receptors were incubated with (■) or without (□) carbachol for 1 h. Cells were then washed and treated with cyclized BCM (50 nM) for 5 min as described in section 2.4.2 "*Receptor recycling assay*". Cells were then incubated for various periods of time for up to 90 minutes at 37 ^{o}C and then used in intact, whole cell [^{3}H]NMS binding assays as described in section 2.5 "*Receptor binding assays*". Each data point represents the mean ± S.E.M. of three to five experiments conducted in triplicate.

Table 2: Comparison of the recovery of specific [^{3}H]NMS binding for M_1-M_5 receptors in untreated and carbachol (1 mM) treated cells[a].

Receptor Subtype/ Condition[b]	Amount internalized[c] (fmol/mg protein)	Initial rate[d] (% of control min^{-1})	Recovery of specific [^{3}H]NMS binding[c] (% of control)
M_1 (5)			
Untreated		0.55 ± 0.15	16.2 ± 1.6
1 h	262.8 ± 42.7	1.17 ± 0.13[f]	22.8 ± 3.7[f]
M_2 (3)			
Untreated		0.28 ± 0.03	12.1 ± 0.6
1 h	204.9 ± 3.0	0.41 ± 0.03	14.2 ± 0.1
M_3 (3)			
Untreated		0.16 ± 0.01	13.2 ± 0.2
1 h	186.4 ± 27.4	0.61 ± 0.07[f]	20.5 ± 0.9[f]
M_4 (3)			
Untreated		0.42 ± 0.07	22.9 ± 1.7
1 h	364.2 ± 23.0	1.30 ± 0.21[f]	32.2 ± 1.6[f]
M_5 (3)			
Untreated		0.27 ± 0.10	16.6 ± 5.3
1 h	96.1 ± 2.1	1.67 ± 0.11[f]	47.1 ± 7.3[f]

[a] Data from Figure 4.

[b] Number of experiments are shown in parenthesis and the duration of carbachol treatment (1 mM) is indicated.

[c] The amount of receptor internalized during carbachol treatment was calculated by subtracting specific [^{3}H]NMS binding for carbachol treated control cells from that obtained for untreated control cells (see section 2.4.2, "*Receptor recycling assay"*).

[d] The initial rate was determined for untreated and carbachol treated cells by fitting data shown in Figure 4 to a single-phase association equation (see section 2.7, "*Data analysis*").

[e] Recovery of specific [^{3}H]NMS binding was calculated by subtracting specific binding at 0 min after BCM treatment from that at 90 min after BCM treatment.

[f] Significantly different from untreated cells ($P < 0.05$) as determined using a paired Student's *t* test (two tailed).

3.1.3 Comparison of M_1-M_5 receptor downregulation

[^{3}H]QNB is a membrane permeable muscarinic receptor selective antagonist and binds to both the cell surface and intracellular muscarinic receptors. Hence [^{3}H]QNB binding was used to determine the total amount of muscarinic receptors expressed in CHO cells. In the current investigation, [^{3}H]QNB was used to assess the change in total M_1-M_5 receptor expressed in intact, whole CHO cells after 24 h carbachol treatment (i.e., receptor downregulation) (see section 2.4.3 "*Receptor downregulation assay*"). To determine whether [^{3}H]QNB can permeate intracellular compartments containing muscarinic receptors, CHO M_1 cells were incubated in the absence and presence of carbachol (1 mM) for 24 h. Untreated and carbachol-treated cells were ruptured using a hypotonic buffer and the resulting membranes were washed and pelleted. Untreated and carbachol treated membranes were then used in [^{3}H]QNB binding assays as described in section 2.5.1 "*Filtration receptor binding assays*". We found that 24 h carbachol treatment caused a 78% reduction in specific [^{3}H]QNB binding in carbachol treated membranes compared to untreated membranes. This reduction was comparable to that observed in intact, whole cell [^{3}H]QNB binding assays conducted on CHO cells expressing M_1 receptors after 24 h carbachol treatment (1 mM) (see Figure 5A and Table 3). These data suggest that [^{3}H]QNB permeates intracellular compartments in intact, whole CHO cells. Consequently, intact, whole cell binding assays were used to measure M_1-M_5 receptor downregulation elicited to 24 h carbachol treatment.

In downregulation assays, CHO cells expressing M_1-M_5 receptors were incubated with carbachol (1 mM) for 24 h. Residual receptors were measured using a single concentration of [^{3}H]QNB (see section 2.5 "*Receptor binding assays*"). Following carbachol treatment, specific [^{3}H]QNB binding in CHO cells expressing M_1, M_2, M_3, M_4 and M_5 receptors decreased by 72.2 ± 3.6%, 40.9 ± 2.0%, 46.5 ± 2.0%, 54.9 ± 0.8% and 50.0 ± 7.6%, respectively (Figure 5 and Table 3).

Specific [^{3}H]NMS binding in intact, whole CHO cells expressing M_1-M_5 receptors after 24 h of carbachol treatment (1 mM) was also determined. In CHO cells expressing M_1, M_2, M_3,

M_4 and M_5 receptors, specific [^{3}H]NMS binding decreased 86.6 ± 1.6%, 84.3 ± 0.6%, 63.4 ± 1.5%, 79.9 ± 0.9% and 75.7 ± 2.2%, respectively (Figure 5 and Table 2). When comparing these [^{3}H]NMS binding data with the data in Figure 4, it can be seen that the loss of cell surface M_1, M_3 and M_5 receptors consists of two components. A rapid component occurs during a short-term treatment with carbachol, and is nearly complete in approximately 4.5 half-times (1-3.5 h) (Figure 1A, C, and E). Longer treatment with carbachol (24 h), however, caused a further loss of M_1, M_3 and M_5 receptors (Figure 5A, C, and E). This slower component was not apparent with M_2 and M_4 receptors (see Figures 1B and D, 5B and D).

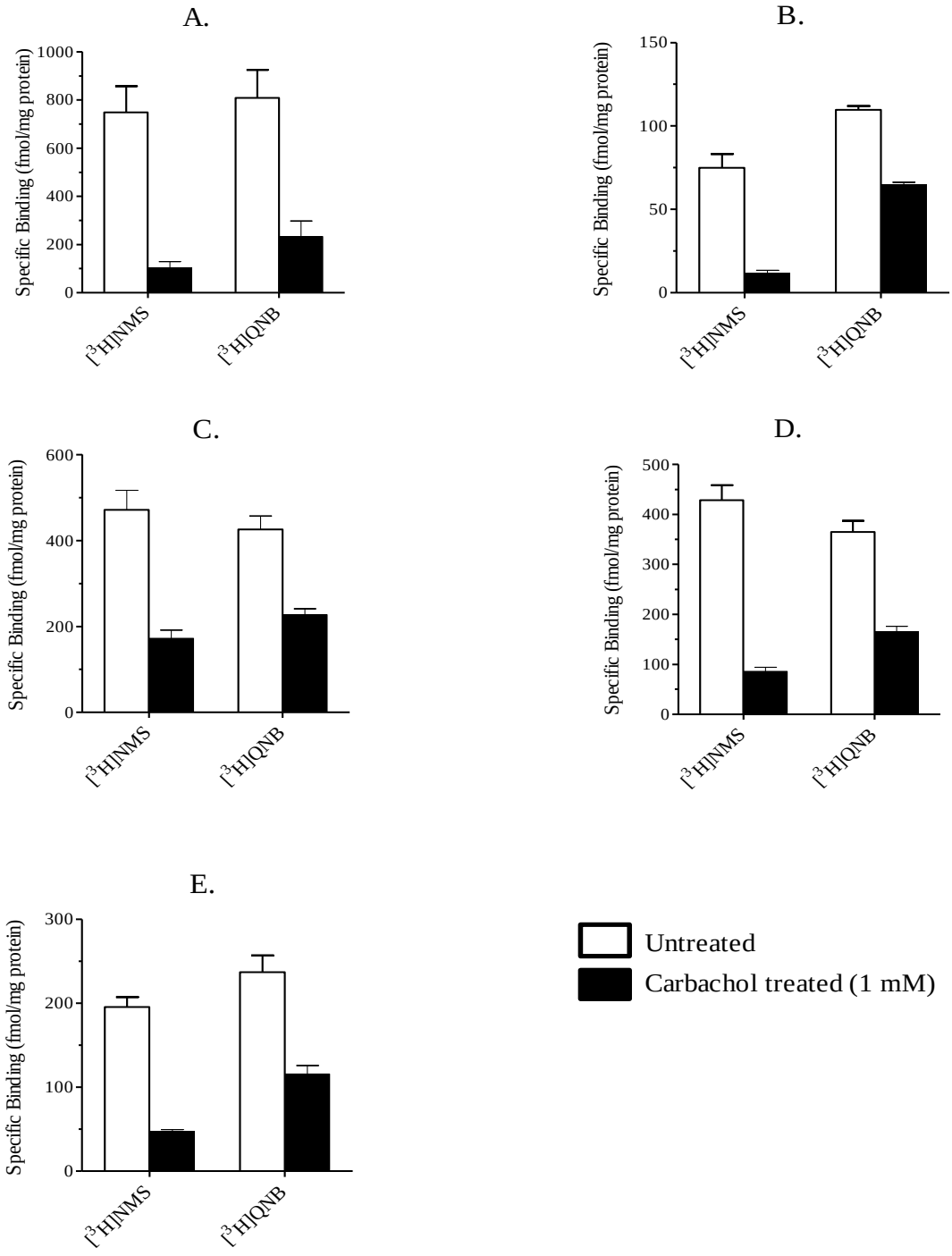

Figure 5. Downregulation of M_1-M_5 receptors. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D) or M_5 (E) receptors were incubated in the absence (open bars) and presence (closed bars) of carbachol (1 mM) for 24 h at 37 ^{o}C. Cells were washed and then used in intact, whole cell binding assays using either [^{3}H]NMS or [^{3}H]QNB as described in section 2.5 "*Receptor binding assays*". Each bar represents the mean ± S.E.M. of three experiments conducted in triplicate.

Table 3: Comparison of the specific [^{3}H]NMS and [^{3}H]QNB binding for M_1-M_5 receptors in CHO cells incubated in the absence and presence of carbachol (1 mM) 24 hours[a].

Receptor subtype/ Condition[b]	Specific [^{3}H]NMS binding (fmol/mg protein)	Specific [^{3}H]NMS binding after treatment (%)[c]	Specific [^{3}H]QNB binding (fmol/ mg protein)	Specific [^{3}H]QNB binding after treatment (%)[d]
M_1 (3)				
Untreated	748.5 ± 109.2		808.1 ± 116.7	
24 h	103.2 ± 26.6	13.4 ± 1.6[e]	233.0 ± 64.6	27.8 ± 3.6[f]
M_2 (3)				
Untreated	74.9 ± 8.3		109.8 ± 2.3	
24 h	11.8 ± 1.6	15.7 ± 0.6[g]	64.9 ± 1.4	59.1 ± 0.1
M_3 (3)				
Untreated	471.0 ± 45.9		425.6 ± 31.1	
24 h	172.2 ± 18.8	36.6 ± 1.5[h]	227.2 ± 13.4	53.5 ± 2.0
M_4 (3)				
Untreated	428.2 ± 30.3		364.4 ± 22.6	
24 h	86.0 ± 8.1	20.1 ± 0.9	164.6 ± 11.5	45.1 ± 0.8
M_5 (3)				
Untreated	195.4 ± 12.1		237.1 ± 20.0	
24 h	47.0 ± 2.5	24.3 ± 2.2	115.9 ± 10.0	50.0 ± 7.6

[a] Data from Figure 5.

[b] Number of experiments are shown in parenthesis.

[c] The specific [^{3}H]NMS binding remaining after 24 h carbachol treatment (1 mM) differed significantly across the receptor subtypes ($p < 0.0001$) as determined by one-way ANOVA.

[d] The specific [^{3}H]QNB binding remaining after 24 h carbachol treatment (1 mM) differed significantly across the receptor subtypes ($p < 0.01$) as determined by one-way ANOVA.

[e] Tukey post-hoc comparisons indicate that the [^{3}H]NMS binding remaining in CHO M_1 cells is significantly different from M_3 ($p < 0.001$) and M_5 ($p < 0.01$) receptor CHO cells.

[f] Tukey post-hoc comparisons indicate that the [^{3}H]QNB binding remaining in CHO M_1 cells is significantly different from M_2 ($p < 0.01$), M_3 ($p < 0.01$) and M_5 ($p < 0.05$) receptor CHO cells.

[g] Tukey post-hoc comparisons indicate that the [^{3}H]NMS binding remaining in CHO M_2 cells is significantly different from M_3 ($p < 0.001$) and M_5 ($p < 0.05$) receptor CHO cells.

[h] Tukey post-hoc comparisons indicate that the [^{3}H]NMS binding remaining in CHO M_3 cells is significantly different from M_4 ($p < 0.001$) and M_5 ($p < 0.001$) receptor CHO cells.

3.2 Characterization of effect of proteasomal and lysosomal inhibitors on the downregulation of M_1-M_5 receptors

3.2.1 Comparison of the extent of carbachol-induced downregulation for various times of incubation with carbachol (1 mM)

We wanted to determine how the specific [^{3}H]QNB binding of CHO cells expressing muscarinic receptors individually change in response to increasing times of incubation with carbachol. CHO cells expressing M_1-M_5 receptors individually were treated with carbachol (1 mM) for various periods of time and then intact whole cell [^{3}H]QNB binding assay (see section 2.4.5 "*Time-course downregulation assay*") was performed to measure the total amount of muscarinic receptors. Changes in [^{3}H]QNB binding is a measure of change in total amount of receptors. The decrease in [^{3}H]QNB binding in response to carbachol (1 mM) was time-dependent. As seen in figure 6A, over a 24 h treatment with carbachol (1 mM) a significant loss in specific [^{3}H]QNB binding was observed. This loss in [^{3}H]QNB binding represents the amount of M_1 receptors degraded (downregulated) in response to long-term carbachol treatment and at 24 h, more than 70% of M_1 receptors expressed were degraded. For M_2 and M_4 receptors loss of [^{3}H]QNB binding occurred progressively till 6 and 12 hours of continuous exposure to carbachol respectively and at 24 h a specific [^{3}H]QNB binding of 45 and 32% of control was observed (Figure 6B and D). Similarly, the specific [^{3}H]QNB binding of M_3 receptors decreased in a time-dependent fashion and a specific [^{3}H]QNB binding of 66% of control was measured at 24 h (Figure 6C). In the case of M_5 receptors, the specific [^{3}H]QNB binding decreased rapidly with carbachol addition for up to 3 h of carbachol-treatment, following which the specific [^{3}H]QNB binding declined to 45% of control at 24 h (Figure 6E). In general, a maximal downregulation (loss of [^{3}H]QNB binding) was observed only at 24 h of carbachol treatment.

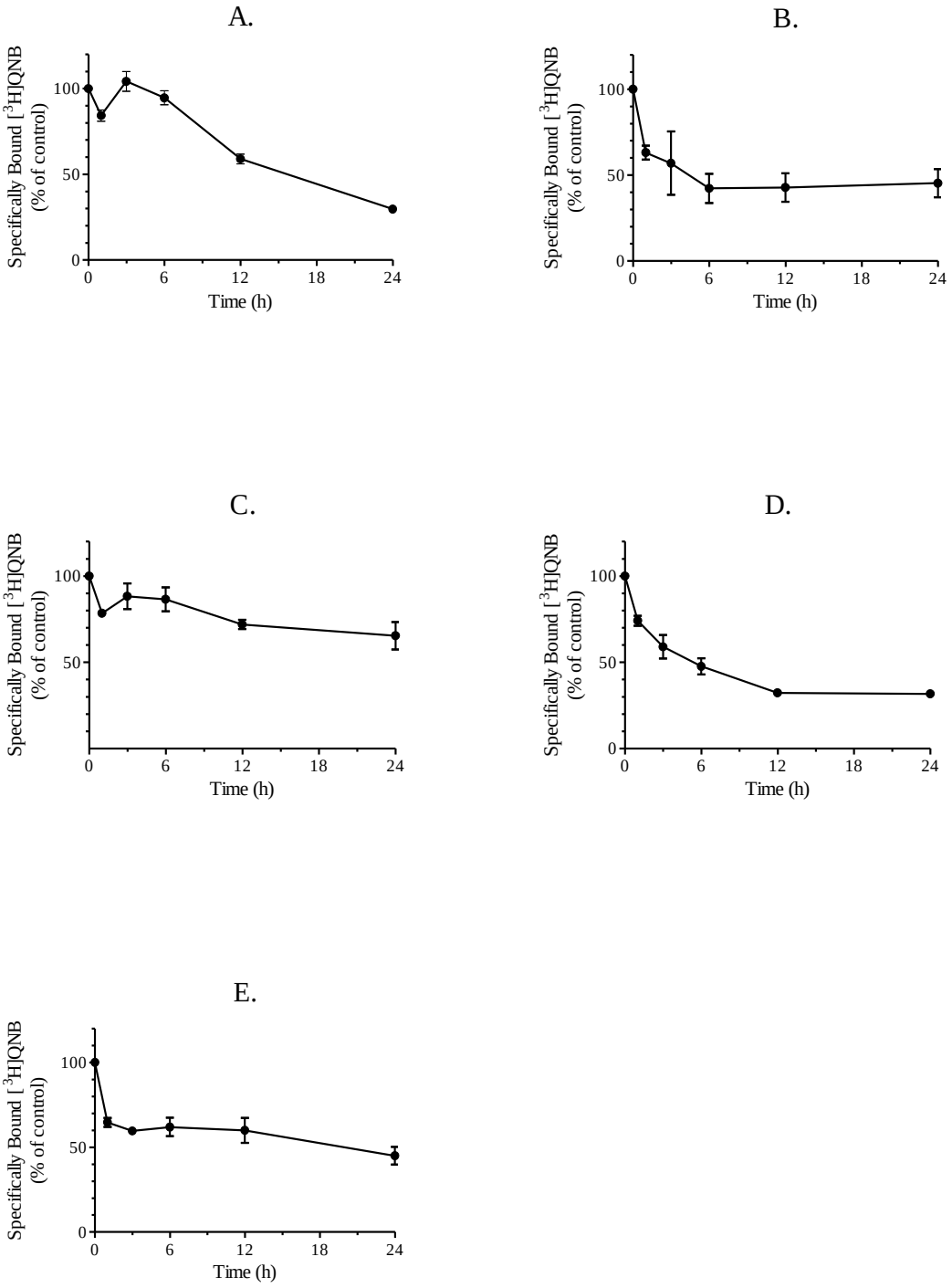

Figure 6. Time-course downregulation of M_1-M_5 receptors. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D), or M_5 receptors (E) were incubated with carbachol (1 mM) for various periods of time for up to 24 h. Cells were washed extensively to remove carbachol and then used in intact, whole cell binding assays using a single concentration of [^{3}H]QNB (1.2 nM) (section 2.5 "*Receptor binding assay*"). Each data point represents the mean ± SEM of three experiments conducted in triplicate.

3.2.2 Comparison of the potency of carbachol-induced downregulation

We also determined the potency of carbachol for eliciting muscarinic receptor downregulation. As described in section 2.4.6 "*Assay to determine the potency of downregulation*", we treated CHO cells expressing M_1-M_5 receptors with equally spaced concentrations of carbachol (1- or 0.5-log unit) for 24 h (M_1 and M_3 receptor expressing cells) or 7 h (M_2, M_4 and M_5 receptor expressing cells). The duration of treatment was determined from data in figure 6. The potency (EC_{50}) of carbachol in downregulating M_1, M_2, M_3, M_4 and M_5 receptors are respectively, 1.1, 2.5, 41, 9.5 and 87 μM respectively. Carbachol is respectively 2, 37, 9 and 79 folds greater in stimulating downregulation in M_1 receptors compared to M_2, M_3, M_4 and M_5 receptors. A carbachol concentration of 1 mM which is at least 10 fold greater than the potency of carbachol for downregulating muscarinic receptors was used in downregulation experiments in the presence of inhibitors.

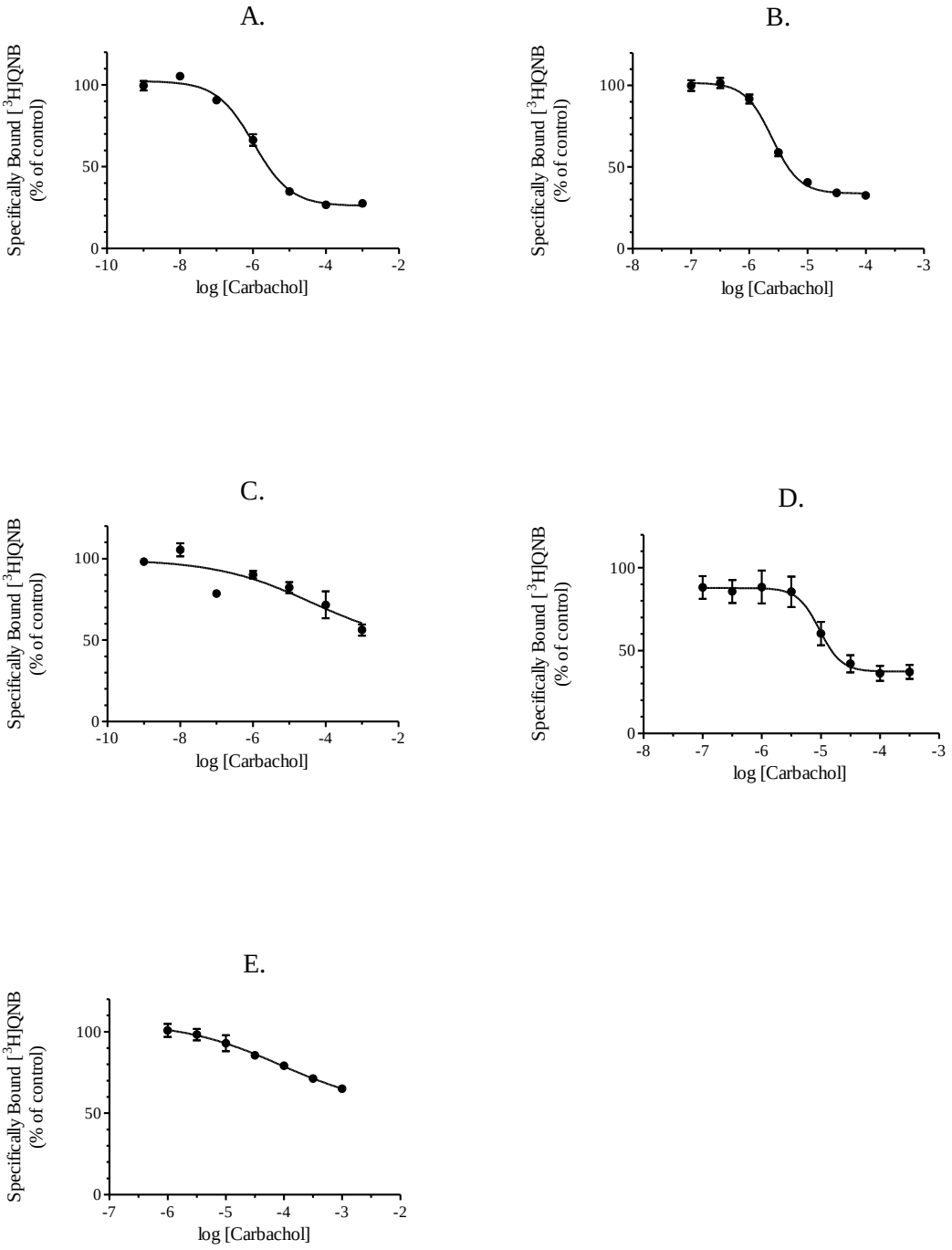

Figure 7. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D), or M_5 receptors (E) were incubated with various concentrations of carbachol. Cells were washed extensively to remove carbachol and then used in intact, whole cell binding assays using a single concentration of [^{3}H]QNB (1.2 nM) as described in section 2.5 "*Receptor binding assays*". Each data point represents the mean ± SEM of three experiments conducted in triplicate.

3.2.3 Comparison of the effect of proteasomal inhibitors on the downregulation of M_1-M_5 receptors

We wanted to determine whether muscarinic receptors are downregulated via a proteasomal mechanism. To investigate the potential role of the proteasomes in downregulating muscarinic receptors, we determined whether the proteasomal inhibitors PSI, MG-132 and lactacystin prevent carbachol-induced downregulation. CHO cells expressing M_1-M_5 receptors individually were treated with various proteasomal inhibitors (PSI, MG-132 and lactacystin) 30 minutes before addition of carbachol. PSI and MG-132 inhibit the chymotrypsin-like activity of 20S proteasome catalytic subunit, while lactacystin acts irreversibly on the catalytical subunit of 20S proteasome inhibiting chymotrypsin and trypsin-like activities (Myung et al., 2001). The cells were then treated with carbachol (1 mM) for 24 h in the continued presence of inhibitors. Treatment of CHO cells expressing muscarinic receptors with proteasomal inhibitors increased [^{3}H]QNB binding compared to vehicle controls. For example, treatment of CHO cells expressing M_1, M_2, M_3, M_4 and M_5 receptors with proteasomal inhibitor PSI in the absence of carbachol caused respectively 1.3-, 1.7-, 1.1-, 1.5- and 1.5- fold increase in the total receptor binding compared to the total binding in the same cells treated with vehicle control (Table 4). The loss of specific [^{3}H]QNB binding (downregulation) for M_1 and M_3 receptors were significantly affected when the carbachol treatment was performed in the continued presence of proteasomal inhibitors PSI, MG-132 and lactacystin (see Figure 8, Table 4). Proteasomal inhibitors MG-132 and lactacystin significantly decreased the downregulation of M_4 receptors, while proteasomal inhibitors PSI and lactacystin had a significant inhibition on the downregulation of M_5 receptors (see Figure 8, Table 4). There was no reduction in the downregulation of M_2 receptors in the continued presence of any of the proteasomal inhibitors used in our study. Instead, carbachol-induced downregulation of M_2 receptors increased significantly in the presence of proteasomal inhibitors (see Figure 8, Table 4). The inhibition of downregulation of muscarinic receptors by proteasomal inhibitors is specific. CHO cells stably expressing M_1 receptors were pre-treated

with various concentrations of proteasomal inhibitor PSI for 30 minutes and then incubated with or without carbachol (1 mM) for 24 h in the continued presence of PSI. The inhibition of downregulation of M_1 receptors by proteasomal inhibitor PSI increased with increasing concentrations of PSI. When CHO cells expressing M_1 receptors were treated with 0.05, 0.25, 0.5, 5 and 15 μM of PSI, we observed 1.5, 18, 57, 59 and 58% of inhibition of downregulation of M_1 receptors in the presence of carbachol for 24 h (data not shown).

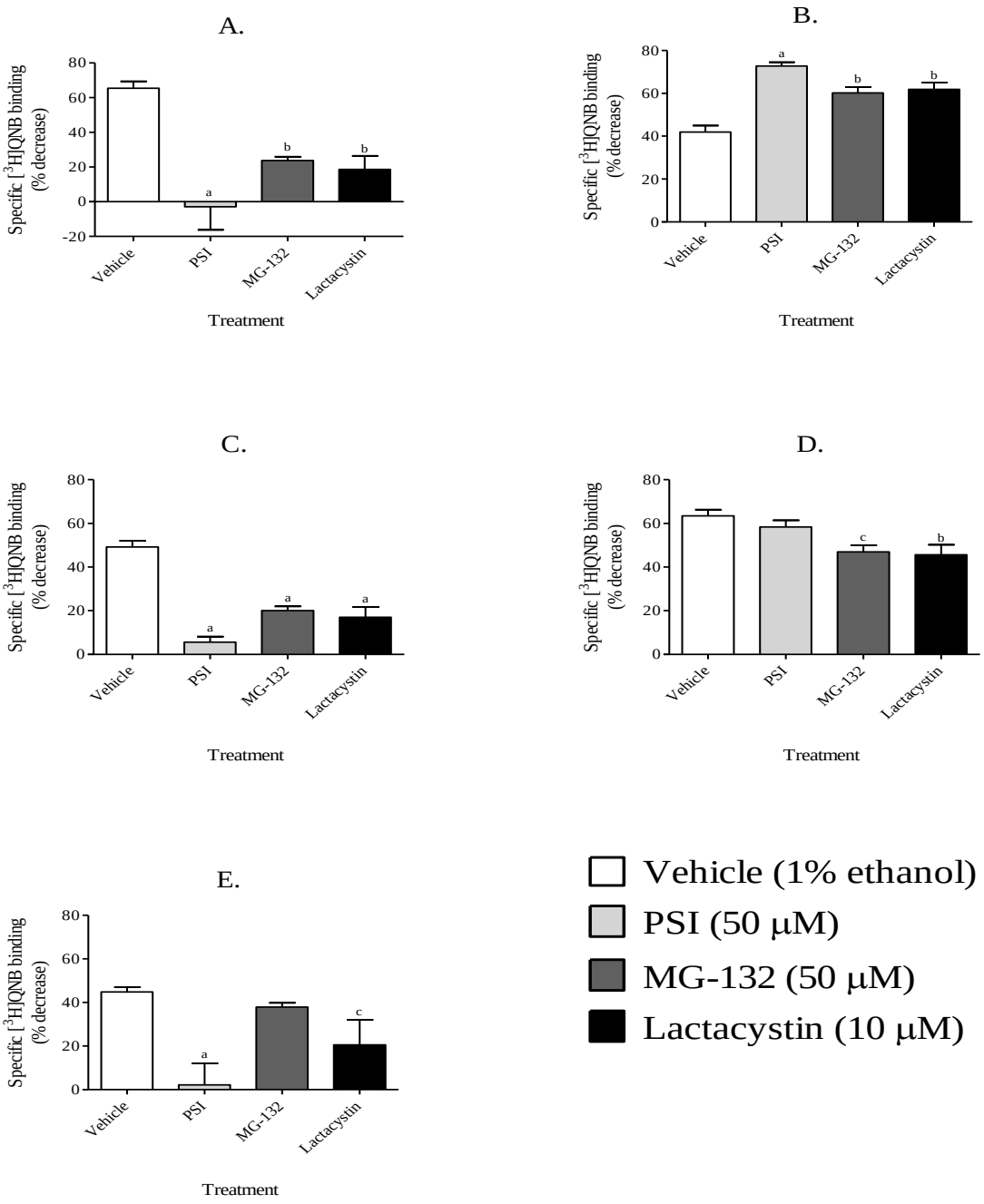

Figure 8. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D), or M_5 receptors (E) were treated with PSI, MG-132, lactacystin or vehicle in the absence and presence of carbachol (1 mM) as described in section 2.4.4 "*Downregulation assay in the presence of inhibitors*". Cells were incubated for 24 h and then washed extensively. Washed cells were then used in intact, whole cell binding assays using a single concentration of [^{3}H]QNB (1.2 nM) (see section 2.5 "*Receptor binding assays*"). Each bar represents the mean ± S.E.M. of three or more experiments performed in triplicate. ^{a}, ^{b}, ^{c} denotes respectively significant differences ($p < 0.001$), ($p < 0.01$)

and ($p < 0.05$) from vehicle treatment as determined using a one-way ANOVA with Dunnett's post-hoc test.

Table 4: Comparison of the specific [^{3}H]QNB binding for M_1-M_5 receptors in CHO cells incubated in the absence and presence of carbachol (1 mM, 24 hours) for vehicle and proteasomal inhibitor pre-treatments[a].

Receptor Subtype	Specifically bound [^{3}H]QNB (fmol/ mg protein)[b]			
	Vehicle (1% ethanol)	PSI (50 µM)	MG-132 (50 µM)	Lactacystin (10 µM)
M_1				
Untreated	1073 ± 89 (7)	1527 ± 109 (4)	1431 ± 179 (3)	1649 ± 235 (3)
24 h Carbachol	374 ± 56	1586 ± 252	1098 ± 165	1333 ± 222
M_2				
Untreated	233 ± 26 (6)	465 ± 85 (3)	415 ± 64 (3)	404 ± 32 (3)
24 h Carbachol	138 ± 20	127 ± 28	146 ± 38	153 ± 15
M_3				
Untreated	851 ± 75 (6)	1002 ± 88 (3)	964 ± 80 (3)	1157 ± 146 (3)
24 h Carbachol	426 ± 25	949 ± 103	772 ± 74	947 ± 64
M_4				
Untreated	657 ± 102 (6)	1014 ± 72 (3)	1019 ± 84 (3)	1294 ± 187 (3)
24 h Carbachol	232 ± 27	419 ± 26	546 ± 74	694 ± 85
M_5				
Untreated	429 ± 38 (7)	672 ± 66 (4)	626 ± 60 (3)	1039 ± 76 (4)
24 h Carbachol	238 ± 25	638 ± 20	387 ± 28	847 ± 182

[a] Data from figure 8

[b] Numbers within parenthesis represent the number of experiments performed in triplicates for each treatment

3.2.4 Comparison of the effect of lysosomal inhibitors on the downregulation of M_1-M_5 receptors

We wanted to determine whether muscarinic receptors are downregulated via a lysosomal mechanism. To investigate the potential role of the lysosomes in downregulating muscarinic receptors, we determined whether the lysosomal inhibitors NH_4Cl and E64-d prevent carbachol-induced downregulation. CHO cells expressing individually M_1-M_5 receptors were treated with lysosomal inhibitors (NH_4Cl and E64-d) 30 minutes prior to addition of carbachol. Following the treatment with lysosomal inhibitors, cells were incubated with or without carbachol for 24 h in the continued presence of lysosomal inhibitors. The carbachol-dependent percent decrease in the specific [^{3}H]QNB binding of the cells treated with lysosomal inhibitors (NH_4Cl, E-64-d) was similar to those cells treated with vehicle control, for M_1, M_3 and M_5 receptors (see Figure 9A,C and E). However, carbachol-stimulated downregulation of M_2 and M_4 receptors was significantly decreased when treated with NH_4Cl (see Figure 9B and D). Further, the carbachol-stimulated downregulation of M_2 and M_4 receptors remained unchanged in response to E-64-d pretreatment. Moreover the inhibition of downregulation of muscarinic receptors by lysosomal inhibitors is specific. CHO cells stably expressing M_2 receptors were pre-treated with various concentrations of lysosomal inhibitor NH_4Cl for 30 minutes and then incubated with or without carbachol (1 mM) for 24 h in the continued presence of NH_4Cl. With increasing concentrations of NH_4Cl, we observed a decrease in M_2 receptor downregulation. When CHO cells expressing M_2 receptors were treated with 0.1, 1 and 10 mM concentrations of NH_4Cl, the inhibition of carbachol-induced downregulation of M_2 receptors were observed to be 13, 21 and 67% at 24 h (data not shown).

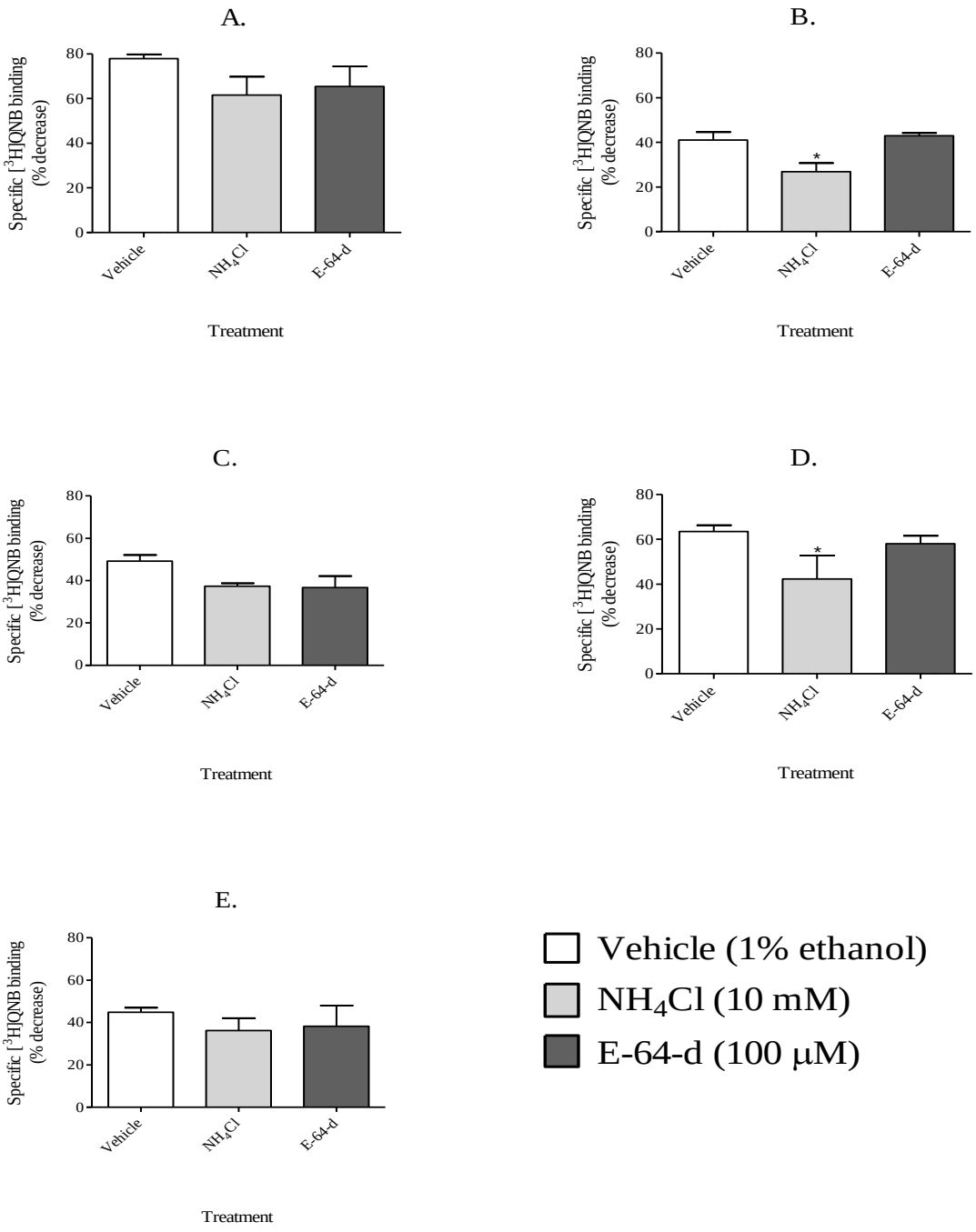

Figure 9. CHO cells stably expressing M_1 (A), M_2 (B), M_3 (C), M_4 (D), or M_5 receptors (E) were treated with NH_4Cl, E-64-d or vehicle in the absence and presence of carbachol (1 mM) as described in 2.4.4 "*Downregulation assay in the presence of inhibitors*". Cells were incubated for 24 h and then washed extensively. Washed cells were then used in intact, whole cell binding assays using a single concentration of [^{3}H]QNB (1.2 nM) (see section 2.5 "*Receptor binding assays*"). Each bar represents the mean ± S.E.M. of three or more experiments performed in triplicate. * Denotes significant differences from vehicle treated condition ($p < 0.05$) as determined using a one-way ANOVA with Dunnett's post-hoc test.

Table 5: Comparison of the specific [^{3}H]QNB binding for M_1-M_5 receptors in CHO cells incubated in the absence and presence of carbachol (1 mM) 24 hours for vehicle and lysosomal inhibitor pre-treatments[a].

Receptor Subtype	Specifically bound [^{3}H]QNB (fmol/mg protein)[b]		
	Vehicle (1% ethanol)	NH_4Cl (10 mM)	E-64-d (100 µM)
M_1			
Untreated	1028 ± 75 (7)	887 ± 107 (3)	937 ± 78 (4)
24 h Carbachol	233 ± 35	335 ± 66	344 ± 124
M_2			
Untreated	198 ± 31 (6)	314 ± 58 (3)	83 ± 5 (3)
24 h Carbachol	115 ± 22	231 ± 49	47 ± 2
M_3			
Untreated	851 ± 75 (6)	770 ± 71 (3)	809 ± 52 (3)
24 h Carbachol	426 ± 25	484 ± 55	507 ± 16
M_4			
Untreated	657 ± 102 (6)	562 ± 122 (3)	319 ± 48 (3)
24 h Carbachol	232 ± 27	302 ± 23	131 ± 7
M_5			
Untreated	429 ± 38 (7)	300 ± 12 (3)	404 ± 67 (4)
24 h Carbachol	238 ± 25	193 ± 25	250 ± 67

[a] Data from figure 9

[b] Numbers within parenthesis represent the number of experiments performed in triplicates for each treatment

3.3 Characterization of domains in the third intracellular loop and C-terminal tail of M_1 receptor

3.3.1 Effect of deletion of the amino acids within regions of third intracellular loop or C-terminal tail on the binding of M_1 receptors

We wanted to determine whether the deletions made in the i3 loop or C terminal tail of muscarinic receptors affected the ability of M_1 receptors to bind muscarinic receptor antagonist [^{3}H]NMS. Hence, we made deletions in the i3 loop and C-terminal tail of the M_1 receptor using site-directed mutagenesis and created CHO cells stably expressing M_1 del 276-282 or M_1 del 447-459 receptors. Saturation binding assays were performed and equilibrium dissociation constant K_D was estimated for CHO cells stably expressing i3 loop or C terminal tail deletion mutants and compared with K_D value for wild-type M_1 receptors. We found that the equilibrium dissociation constants for wild-type and mutant receptors were comparable (Figure 10, Table 6) and the deletion mutations did not affect the affect the affinity of M_1 receptors.

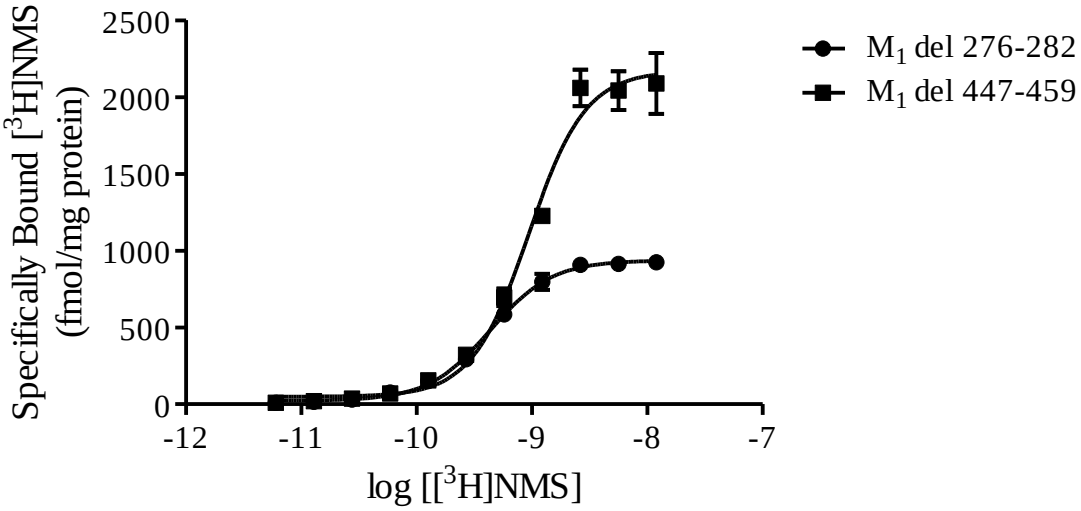

Figure 10. Effect of deletion of amino acids in the i3 loop or C terminal tail of M_1 receptors on [^{3}H]NMS binding. Intact, whole cell [^{3}H]NMS binding assays were performed on CHO cells stably expressing muscarinic M_1 del 276-282 or M_1 del 447-459 receptors (see section 2.5 "*Receptor binding assays*"). Each data point represents the mean ± S.E.M. of two experiments conducted in triplicate.

Table 6: Comparison of affinity and binding capacity for wild-type and mutant muscarinic M_1 receptors [a]

Receptor [b]	K_D (nM) [c]
M_1 wild-type (2)	0.61 ± 0.02[*]
M_1 del 276-282 (2)	0.43 ± 0.001
M_1 del 447-459 (2)	0.94 ± 0.06

[*] adapted from Sawyer et al., 2010

[a] Data from Figure 10.
[b] Number of experiments are shown in parenthesis.
[c] K_D was determined by fitting data shown in Figure 10 to a non-linear regression equation.

3.3.2 Effect of the deletion of amino acids within regions of third intracellular loop or C-terminal tail on the signaling of M_1 receptors.

We wanted to determine the functional role of mutant receptors. To estimate the functional response of the deletion mutants, phosphoinositide hydrolysis assays were performed. CHO cells expressing either M_1 wild-type, M_1 del 276-282 or M_1 del 447-459 receptors were incubated with [^{3}H]myo-inositol for 18h. The cells were stimulated with equally spaced concentrations (0.5-log unit) of carbachol for 30 minutes and then [^{3}H]inositol phosphates were counted. The potency (EC_{50}) and maximal response (E_{max}) for the wild-type and mutant receptors were estimated using a non-linear regression analysis. Deletion of amino acids 276-282 in the i3 loop region of M_1 receptors caused respectively, 3.2- and 1.5- fold increase in the EC_{50} and E_{max} values compared to wild-type M_1 receptors (Figure 11 and Table 7). The EC_{50} value of M_1 del 447-459 receptors were 5.8 fold greater than that of M_1 wild-type receptors, while the E_{max} value of M_1 del 447-459 receptors is only 53% of that of wild-type M_1 receptors (Figure 11 and Table 7). From the data obtained, it is clear that the mutant M_1 receptors were functional even though potency and signaling were affected differently by deletion mutations.

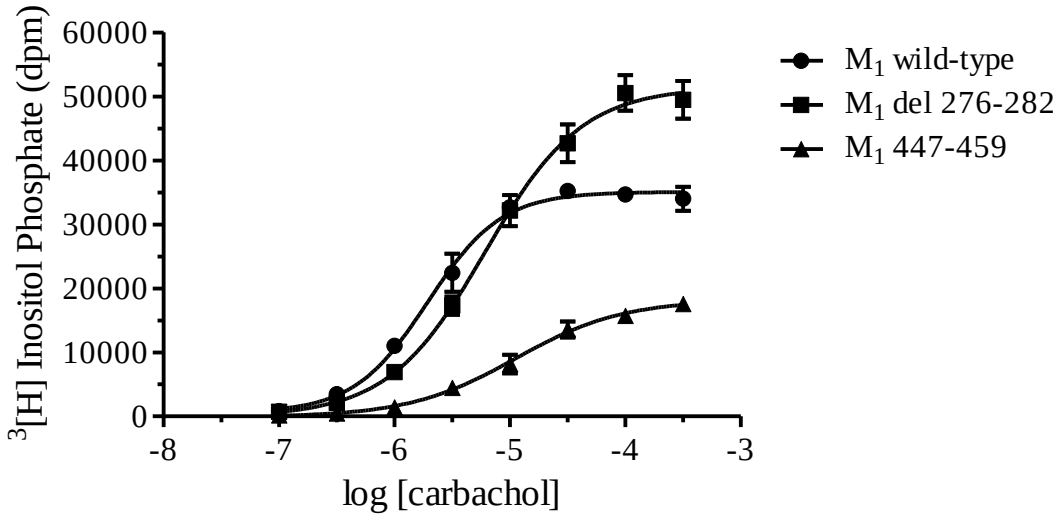

Figure 11. The effect of deletion of amino acids within regions of i3 loop or C terminal tail on carbachol-mediated phosphoinositide hydrolysis. CHO cells stably expressing muscarinic M_1 del 276-282 or M_1 del 447-459 receptors were incubated with 0.2 µM [^{3}H]myo-inositol for 18 h and then phosphoinositide hydrolysis assay was performed as described in section 2.6 "*Phosphoinositide hydrolysis assays*". Each data point represents the mean ± S.E.M. of three experiments conducted in triplicate.

Table 7: Comparison of potency and E_{max} for wild-type and mutant muscarinic M_1 receptors[a]

Receptor[b]	pEC_{50}[c]	E_{max}[c]
M_1 wild-type (3)	5.7 ± 0.2[d]	34508 ± 1784[e]
M_1 del 276-282 (3)	5.2 ± 0.2	51471 ± 3136
M_1 del 447-459 (3)	4.9 ± 0.1	18314 ± 1673

[a] Data from Figure 11.

[b] Number of experiments are shown in parenthesis.

[c] pEC_{50} and E_{max} were determined by fitting data shown in Figure 11 to a non-linear regression equation.

[d] significantly different from EC_{50} of M_1 del 276-282 ($p < 0.05$) and M_1 del 447-459 ($p < 0.01$) receptors as determined using a one-way ANOVA with Dunnett's post-hoc test

[e] significantly different from E_{max} of M_1 del 276-282 ($p < 0.001$) and M_1 del 447-459 ($p < 0.01$) receptors as determined using a one-way ANOVA with Dunnett's post-hoc test

3.3.3 Comparison of the kinetics and extent of internalization of wild-type and mutant M_1 receptors:

We determined if the kinetics and extent of agonist-induced internalization of M_1 receptors was affected by deletion mutations introduced in the i3 loop or C-terminal tail. CHO cells expressing M_1 wild-type receptors, M_1 del 276-282 or M_1 del 447-459 receptors were incubated with the muscarinic receptor selective-agonist carbachol (1 mM) for various times up to four h and then receptor binding at the cell surface was measured using [^{3}H]NMS (1.6 nM). The internalization of all the three receptors (M_1 wild-type, M_1 del 276-282 and M_1 del 447-460 receptors) were consistent with a single phase exponential decay process (Figure 12).

The half-time and plateau for M_1 wild-type receptor was 46.33 min and 54.58% of control respectively. The half time for muscarinic M_1 del 276-282 receptor (63.77 min and 52.43% of control) was nearly1.4 fold greater than that of muscarinic M_1 wild-type receptor while the plateau of internalization is same as that of wild-type receptor. The half time for muscarinic M_1 del 447-459 receptor (24.70 min and 55.62 % of control) was 53% of the half-time of muscarinic M_1 wild-type receptor while the plateau of internalization is same as that of wild-type receptor (Figure 12, Table 8). There were no significant differences in the rate constant and plateaus of internalization between the wild-type and mutant M_1 receptors suggesting that carbachol-induced internalization was unaffected by the mutation. This suggests that the agonist-dependent internalization of M_1 receptors remain unaffected by the deletion mutations.

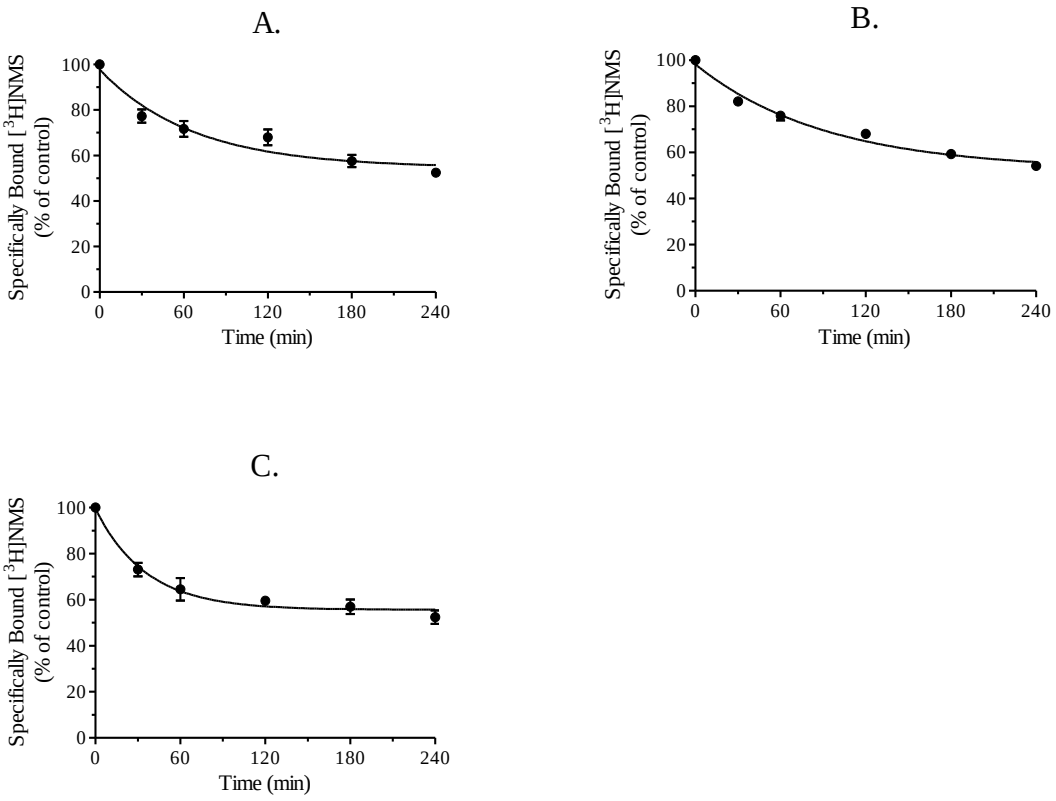

Figure 12. Comparison of internalization of wild-type and mutant M_1 receptors. CHO cells stably expressing muscarinic M_1 wild-type (A), M_1 del 276-282 (B), or M_1 del 447-459 (C) receptors were incubated with carbachol (1 mM) for various periods of time for up to 240 min at 37^{o}C. Cells were then used in intact, whole cell [^{3}H]NMS binding assays (see section 2.5 "*Receptor binding assays*"). Each data point represents the mean ± S.E.M. of three experiments conducted in triplicate.

Table 8: Comparison of specific [^{3}H]NMS binding and rate constants for wild-type and mutant muscarinic M_1 receptor internalization[a].

Receptor[b]	Specific [^{3}H]NMS binding in untreated cells (fmol/mg protein)	Specific [^{3}H]NMS binding in cells treated with carbachol for 240 min (fmol/mg protein)	K (min^{-1})[c]	Plateau (% of control)[d]
M_1 wild-type (3)	1395.2 ± 42.5	731.5 ± 34.1	0.015 ± 0.004	54.58 ± 3.43
M_1 del 276-282 (3)	370.7 ± 15.4	200.5 ± 6.7	0.011 ± 0.002	52.43 ± 2.75
M_1 del 447-459 (3)	1454.6 ± 72.0	762.4 ± 51.3	0.028 ± 0.005	55.62 ± 1.91

[a] Data from Figure 12.

[b] Number of experiments are shown in parenthesis.

[c] The rate constant for internalization was determined by fitting data shown in Figure 12 to a single-phase decay equation.

[d] The plateau for internalization was determined by fitting data shown in Figure 12 to a single-phase decay equation.

3.3.4 Comparison of the recovery of wild-type and mutant M_1 receptors in untreated and carbachol-treated cells following incubation with BCM

We wanted to determine if recycling of M_1 receptors was affected by deletion of domains in i3 loop or C-terminal tail. CHO cells stably expressing M_1 wild-type, M_1 del 276-282 or M_1 del 447-459 receptors were used in recycling assays as described in section 2.4.2 " *Receptor recycling assay*". [^{3}H]NMS was used to quantitate the amount of receptors delivered to the plasma membrane in untreated and carbachol-treated CHO cells. Any difference in the initial rate or extent of delivery of muscarinic receptors to the plasma membrane between the untreated and carbachol-treated condition would be a proof of recycling. BCM, an irreversible muscarinic receptor antagonist was used in recycling assays. CHO cells stably expressing M_1 wild-type receptors, M_1 del 276-282 or M_1 del 447-459 receptors were incubated with 50 nM BCM for 5 min. Nearly, 90.4 ± 1.8%, 92.2 ± 2.3% and 82.4 ± 1.2% of M_1 wild-type, M_1 del 276-282 and M_1 del 447-459 receptors, respectively were alkylated.

In CHO cells expressing M_1 wild-type receptors, 1 h carbachol treatment caused a 22% decrease in specific [^{3}H]NMS binding. The initial rate of recovery of [^{3}H]NMS binding in CHO cells expressing M_1 wild-type receptors was 2.5 ± 0.4 fold greater in carbachol treated cells than in untreated cells after BCM alkylation. At 90 min after BCM alkylation, maximal M_1 wild-type receptor recovery was 1.4-fold greater in carbachol treated cells when compared to untreated cells (Figure 13 and Table 9).

In CHO cells expressing M_1 del 276-282 receptors, 1 h carbachol treatment caused 18% decrease in specific [^{3}H]NMS binding while 2 h carbachol treatment caused 30% decrease in specific [^{3}H]NMS binding. In order to achieve a percentile of internalization comparable to 1 h of internalization observed in wild-type receptors, we treated CHO cells expressing M_1 del 276-282 receptors for 2 h before performing recycling assays. The initial rate of recovery of [^{3}H]NMS binding in CHO cells expressing M_1 del 276-282 receptors was nearly the same in both carbachol

treated and untreated cells. At 90 min after BCM alkylation, maximal M_1 del 276-282 receptor recovery of carbachol treated cells was similar to that of untreated cells (Figure 13 and Table 9).

1 h carbachol treatment caused 24% reduction in [^{3}H]NMS binding in CHO cells expressing M_1 del 447-459 receptors. The initial rate of recovery of [^{3}H]NMS binding in CHO cells expressing M_1 del 447-459 receptors was similar in both carbachol treated and untreated cells. At 90 min after BCM alkylation, maximal M_1 del 447-459 receptor recovery of carbachol treated cells was comparable to that of untreated cells (Figure 13 and Table 9).

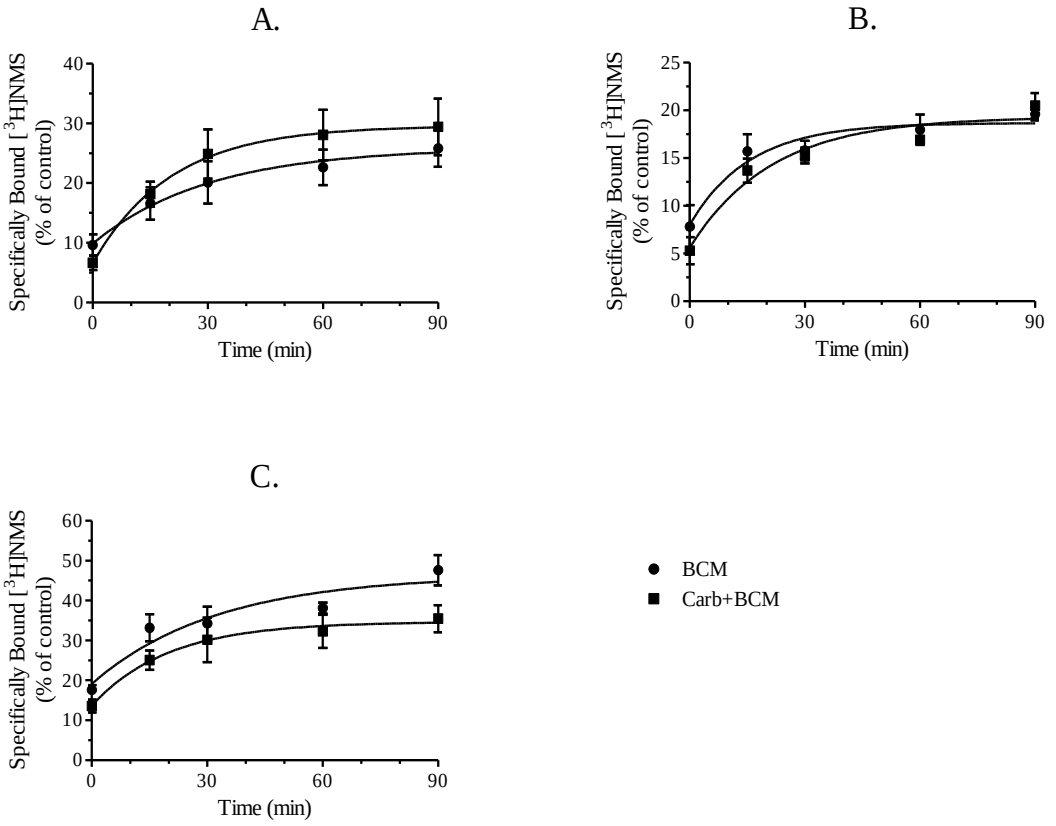

Figure 13**.** Comparison of recycling of wild-type and mutant M_1 receptors. CHO cells stably expressing M_1 wild-type (A), M_1 del 276-282 (B) or M_1 del 447-459 (C) receptors were incubated with (■) or (●) without carbachol. Cells were then washed and treated with cyclized BCM (50 nM) for 5 min. Cells were then incubated for various periods of time for up to 90 minutes at 37^{o}C and then used in intact, whole cell [^{3}H]NMS binding assays (see section 2.5 "*Receptor binding assays*"). Each data point represents the mean ± S.E.M. of three to five experiments conducted in triplicate.

Table 9: Comparison of the recovery of specific [^{3}H]NMS binding for wild-type and mutant M_1 receptors in untreated and carbachol treated cells[a].

Receptor/ Specific Condition[b]	Amount Internalized[c] (fmol/mg protein)	Initial Rate[d] (% of control min^{-1})	Recovery of [^{3}H]NMS binding (% of control)
M_1 wild-type (5)			
Untreated		0.55 ± 0.15	16.2 ± 1.6
1 h	262.8 ± 42.7	1.17 ± 0.13*	22.8 ± 3.7*
M_1 del 276-282 (3)			
Untreated		0.99 ± 0.54	11.8 ± 1.7
2 h	106.8 ± 10.7	0.67 ± 0.23	15.2 ± 0.4
M_1 del 447-459 (3)			
Untreated		1.09 ± 0.46	30.0 ± 3.7
1 h	366.8 ± 84.2	1.03 ± 0.29	21.9 ± 1.9

[a] Data from Figure 13.
[b] Number of experiments are shown in parenthesis and the duration of carabachol (1 mM) is indicated.
[c] The amount of receptor internalized during carbachol treatment was calculated by subtracting specific [^{3}H]NMS binding for carbachol treated cells from that for untreated cells.
[d] The initial rate was determined for untreated and carbachol treated cells by fitting data shown in Figure 13 to a single-phase association equation.
[e] Recovery of specific [^{3}H]NMS binding was calculated by subtracting specific binding at 0 min after BCM treatment from that at 90 min.
* Significantly different from untreated cells ($P < 0.05$) as determined using a paired Student's t-test (two-tailed).

3.3.5 Comparison of downregulation of wild-type and mutant M_1 receptors

We determined the downregulation of M_1 del 276-282 and M_1 del 447-459 receptors and compared with the downregulation of M_1 wild-type receptors in CHO cells. CHO cells expressing wild-type or mutant M_1 receptors were incubated with carbachol (1 mM) for 24 h and then downregulation assay was performed as described in section 2.4.3 "*Receptor downregulation assay*". [^{3}H]NMS and [^{3}H]QNB are membrane-impermeable and membrane-permeable ligands respectively for muscarinic receptors. Hence decrease in [^{3}H]NMS and [^{3}H]QNB binding is a measure of decrease in cell surface and total muscarinic receptors. Following carbachol treatment, specific [^{3}H]NMS binding in CHO cells expressing M_1 wild-type, M_1 del 276-282 and M_1 del 447-459 receptors decreased by 86.6 ± 1.6%, 49.1 ± 2.8% and 23.5 ± 2.3%, respectively. Specific [^{3}H]QNB binding in CHO cells expressing M_1 wild-type, M_1 del 276-282 and M_1 del 447-459

receptors decreased by 72.2 ± 3.6%, 26.4 ± 2.2% and 1.9 ± 11.8% , respectively when incubated with carbachol (1 mM) for 24 h (Figure 14 and Table 10).

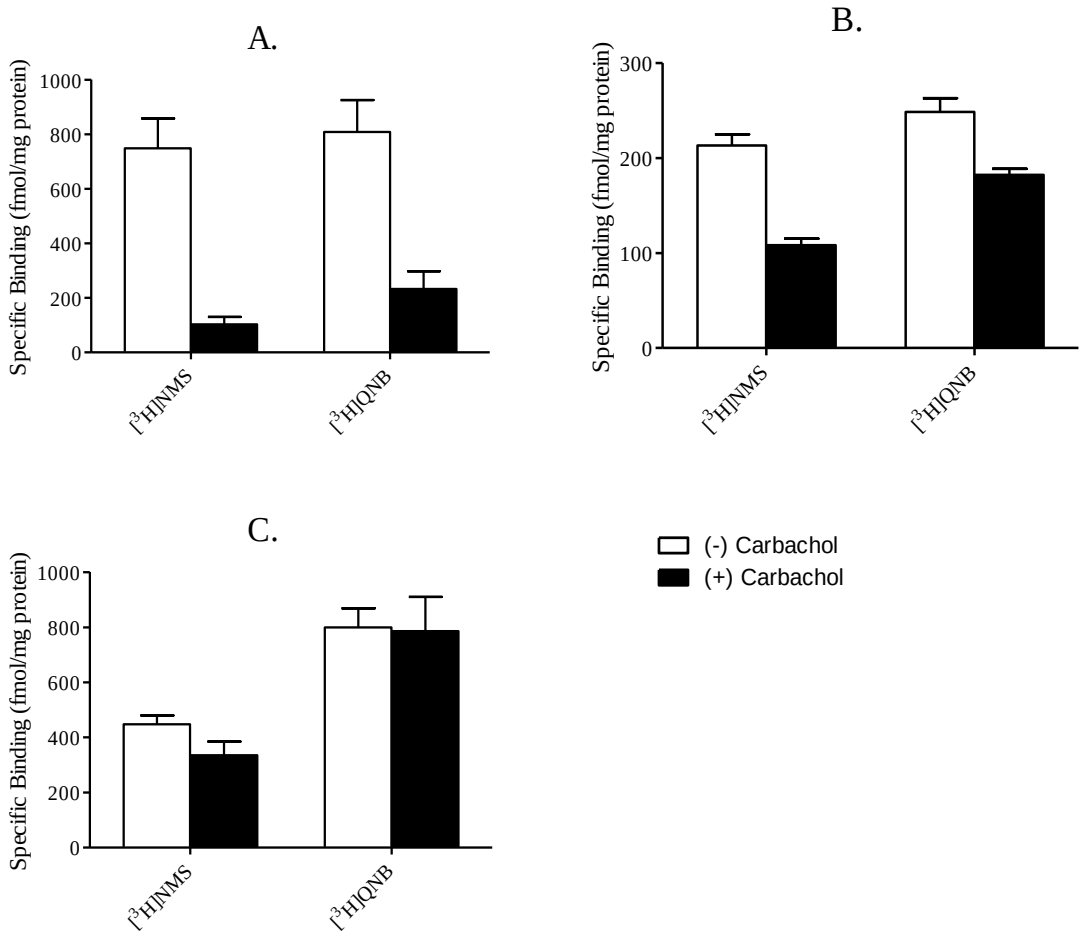

Figure 14. Comparison of downregulation of wild-type and mutant M_1 receptors. CHO cells stably expressing M_1 wild-type (A), M_1 del 276-282 (B) or M_1 del 447-459 (C) receptors were incubated in the absence (open bars) and presence (closed bars) of carbachol (1 mM) for 24 h at 37^{o}C. Cells were washed and then used in intact, whole cell binding assays using either [^{3}H]NMS or [^{3}H]QNB (see section 2.5 "*Receptor binding assays*"). Each bar represents the mean ± S.E.M. of three experiments conducted in triplicate.

Table 10: Comparison of the specific [^{3}H]NMS and [^{3}H]QNB binding for wild-type and mutant M_1 receptors in CHO cells incubated in the absence and presence of carbachol (1 mM) 24 hours[a].

Receptor / Condition[b]	Specific [^{3}H]NMS binding (fmol/mg protein)	Specific [^{3}H]NMS binding after treatment (%)[c]	Specific [^{3}H]QNB binding (fmol/ mg protein)	Specific [^{3}H]QNB binding after treatment (%)[d]
M_1 wild-type (3)				
Untreated	748.5 ± 109.2		808.1 ± 116.7	
24 h	103.2 ± 26.6	13.4 ± 1.6	233.0 ± 64.6	27.8 ± 3.6
M_1 del 276-282 (3)				
Untreated	213.4 ± 11.7		248.7 ± 14.5	
24 h	108.5 ± 6.8	50.9 ± 2.8[c]	182.4 ± 6.3	73.6 ± 2.2[d]
M_1 del 447-459 (5)				
Untreated	447.2 ± 32.6		799.1 ± 69.2	
24 h	335.4 ± 49.4	76.5 ± 2.3[c]	785.5 ± 124.1	98.1 ± 11.8[d]

[a] Data from Figure 14.

[b] Number of experiments is shown in parenthesis.

[c] Significantly different ($P < 0.01$) from M_1 wild-type receptors as determined by one-way ANOVA with Dunnett's post-hoc test.

[d] Significantly different ($P < 0.01$) from M_1 wild-type receptors as determined by one-way ANOVA with Dunnett's post-hoc test.

CHAPTER IV

DISCUSSION

4.1 Characterization of the agonist-dependent internalization, recycling and downregulation of muscarinic receptors

In this investigation, subtype-specific differences in the internalization, recycling and downregulation of M_1-M_5 receptors in CHO cells were observed. Proteins playing a central role in the agonist-induced regulation of GPCRs such as GRKs and arrestins are expressed in many different cell types to similar levels (Attramadal et al., 1992; Komori et al., 1998; Parruti et al., 1993; Shenoy and Lefkowitz, 2003) and hence subtype-specific differences in agonist-dependent internalization, recycling and downregulation exists in other cell types. These differences were unrelated to whether the subtype signaled through $G_{q/11}$ (M_1, M_3 and M_5) or $G_{i/o}$ (M_2 and M_4), suggesting that the subtypes use different mechanisms for internalization, recycling and downregulation. Stable CHO cell lines expressing muscarinic receptors individually to different levels were used in the study and comparison of rates and extents of internalization were studied. Previous studies have shown that the loss of cell surface [^{3}H]NMS binding (internalization) for carbachol treated cells was independent of the plasma membrane receptor expression in U293 cells and JEG-3 cells (Lameh et al., 1992; Goldman et al., 1996). Hence our comparisons of the internalization of M_1-M_5 receptors, which express around 1395 ± 43, 178 ± 13, 549 ± 43, 439 ± 16, 263 ± 32 fmol/ mg of proteins respectively is valid and within the 10 fold range (Table 1).

In the current study in CHO cells while M_1 and M_3 receptors internalized slowly and to a small extent, the M_4 and M_5 receptors internalized to a faster and greater extent (Figure 1). Among the five subtypes studied by us, the M_2 receptor internalized fastest and greatest of all (Figure 1). These observations are generally consistent with those of other investigators working on CHO cells (Koenig and Edwardson, 1996; Tsuga et al., 1998a; Sawyer et al., 2006). The rate and extent of carbachol-induced muscarinic M_5 receptor internalization in CHO cells has not been characterized previously.

Under similar assay conditions in COS-7 cells (i.e., 1 mM carbachol treatment), the half-times for carbachol-induced M_1 and M_5 receptor internalization ($t_{½}$ = 43 min and $t_{½}$ = 12 min, respectively; (Tsuga et al., 1998b)) were comparable to those obtained in the current study. In contrast, the half-time for M_3 receptor internalization in COS-7 cells ($t_{½}$ = 11 min) was 31% of that observed in our study (Tsuga et al., 1998b). More M_1, M_3 and M_5 receptors internalized in CHO cells (32%, 33% and 63% of control, respectively) than in COS-7 cells (10%, 20% and 25% of control, respectively) after 2 h of carbachol treatment (1 mM) (Tsuga et al., 1998b). Tsuga and coworkers, (1998b) also determined that 10 µM and 100 µM carbachol treatment elicited maximal M_2 and M_4 receptor internalization, respectively in COS-7 cells. Under these assay conditions, the half-times for M_2 and M_4 receptor internalization in COS-7 cells ($t_{½}$ = 37 min and 49 min, respectively) were different than those observed in the present study ($t_{½}$ = 8.6 min and 12.8 min, respectively) (Tsuga et al., 1998b). Additionally, M_2 and M_4 receptors internalized to a lesser extent in COS-7 cells (47% and 45% of control, respectively) than in CHO cells (88% and 77% of control, respectively) after two hours of carbachol treatment (Tsuga et al., 1998b). Overall, there are differences in the carbachol-induced internalization of muscarinic receptor subtypes in COS-7 cells, when compared to CHO cells. The rank order for the rate of internalization in CHO cells observed in our study was $M_2 > M_4 > M_5 > M_3 > M_1$ whereas in COS-7 cells it was $M_3 > M_5 > M_2 > M_1 > M_4$ (Tsuga et al., 1998b). Differences in the rank order for internalization of muscarinic receptor subtypes expressed in CHO cells and COS-7 cells could

be due to the differences in the levels of expression of arrestins and GRKs in COS-7 cells and CHO cells (Menard et al., 1996).

We observed a significant amount of internalization in CHO cells expressing muscarinic receptors when treated with agonist carbachol for 1 hour. The amount of internalization at 1 h of carbachol treatment was sufficient to see internalized receptors recycle back to the plasma membrane. Additionally, the recycling kinetics was also independent of the amount of receptors internalized. Increasing the size of internalized receptor pool available for recycling by increasing the incubation time with carbachol had no effect on the rate of recovery, but only increased the extent of recovery (Koenig and Edwardson, 1994a). Further, the extent of carbachol treatment did not significantly affect the rate of recovery of M_2 receptors in HEK293 cells (Roseberry and Hosey 1999). In our study, we identified no significant differences between the rate constants for recovery in the presence of carbachol when internalization of M_5 receptors was increased from 87.9 fmol/mg of protein (corresponding to 1 hour internalization with 1 mM carbachol) to 111.7 fmol/mg of protein (corresponding to 3 hour internalization with 1 mM carbachol) (data not shown). This observation very clearly indicated that the rate constant for recycling did not depend upon the amount of receptors internalized or available to recycle and served as strong evidence in validating our comparison of agonist-induced recovery among the five subtypes of muscarinic receptors.

In our recycling experiments, BCM was used for the alkylation phase because it has a short half-time for alkylation ($t_{½}$ = 33 sec). A 5-min incubation with BCM (50 nM) alkylated 85% or more of muscarinic M_1-M_5 receptors expressed on the plasma membrane of CHO cells (see Figure 3). It is likely that only a few muscarinic receptors traffic to the plasma membrane of CHO cells during this 5 min incubation with BCM. Thus, in recycling experiments for alkylation of muscarinic receptors with BCM, an incubation temperature of 37 ^{o}C was used instead of a temperature (e.g., 15 ^{o}C) that inhibits receptor trafficking. In previous investigations using PrBCM mustard ($t_{1/2}$ for alkylation = 2 min), it was necessary to incubate cells at 15 ^{o}C or 20 ^{o}C

to inhibit receptor trafficking during the 20 to 30 min incubation with PrBCM (Koenig and Edwardson, 1994b; Koenig and Edwardson, 1994a; Koenig and Edwardson, 1996). By using the approach in our study, we considerably reduced the time necessary to alkylate a significant fraction of plasma membrane expressed muscarinic receptor.

In cells not exposed to carbachol, trafficking of receptors to the plasma membrane after BCM treatment may be from the endoplasmic reticulum, and thus are newly synthesized, or from endosomes or both. In previous investigations, pretreatment of cells with protein synthesis inhibitor cycloheximide significantly reduced the rate of [^{3}H]NMS recovery after irreversibly alkylating the cell surface receptors with PrBCM (Koenig and Edwardson, 1994b; Koenig and Edwardson, 1994a; Haddad et al., 1995; Koenig and Edwardson, 1996). These data demonstrate that a significant fraction of the total receptors that trafficked to the plasma membrane after treatment with PrBCM were newly made receptors. Therefore, it is anticipated that newly synthesized muscarinic receptor also makes up a significant fraction of the receptor delivered to the plasma membrane of CHO cells after BCM treatment in untreated cells. In recycling assays, the rank order of the initial rate and extent of receptor delivery after BCM treatment in untreated cells is $M_1 > M_4 > M_2 = M_5 > M_3$ and $M_5 = M_1 > M_4 > M_2 > M_3$, respectively (Table 2 and Figure 4).

In carbachol-treated cells (1 mM, 1 h), the initial rate of recovery of [^{3}H]NMS binding is a measurement of the basal plasma membrane delivery of muscarinic receptors plus the delivery of internalized muscarinic receptor that recycle back to the plasma membrane. Thus a difference in the rate or extent of recovery of specific [^{3}H]NMS binding in carabachol-treated cells versus untreated cells would be an indication of muscarinic receptor recycling. Using this approach, it was found that the M_2 receptor did not recycle and that the rank order of recycling for other receptors was $M_5 > M_4 > M_3 > M_1$ (Table 2). The observation that M_4 receptors recycled to a greater extent than M_3 receptors in CHO cells was consistent with the studies performed by

Koenig and Edwardson, (1996) using a different approach. The recycling of M_1, M_2 and M_5 receptors was not determined in their study (Koenig and Edwardson, 1996).

The downregulation of M_1, M_2 and M_3 receptors observed in our study was comparable to that previously observed in CHO cells (Tsuga et al., 1998a; Shockley et al., 1999). Prior to the current study, the agonist-induced downregulation of the M_5 receptor had not been determined and the downregulation of M_4 receptor had not been characterized in CHO cells. The downregulation of the M_1 receptor in U293 cells was 22% of that observed in our study, suggesting that cell type may influence the extent of muscarinic receptor downregulation (Lameh et al., 1992). In contrast to the difference in M_1 receptor downregulation in CHO and U293 cells, the downregulation of M_4 receptors in AtT-20 cells was comparable to that observed in the present study (Lenz et al., 1994). Further in endogenous brain tissue, the total protein levels of each of the M_1, M_2 and M_4 receptors in acetylcholineesterase knockout mice were reduced approximately 50% compared to the wild type mice suggesting the occurrence of downregulation of muscarinic receptors in response to endogenous acetylcholine (Li et al., 2003).

Treatment with carbachol for 24 h caused a significant reduction in specific [^{3}H]NMS binding of muscarinic receptors. In CHO cells expressing M_2 and M_4 receptors, specific [^{3}H]NMS binding remaining after 24 h carbachol treatment (16% and 20%, respectively) was comparable to that observed after 4 h carbachol treatment (13% and 25%, respectively) (see Tables 1 and 2). This observation suggests that the maximal effect of carbachol on the plasma membrane expression of M_2 and M_4 receptors occurs within a relatively short period of time. In contrast, the percent specific [^{3}H]NMS binding remaining after 24-h carbachol treatment (1 mM) in CHO cells expressing muscarinic M_1 (13%), M_3 (37%) and M_5 (24%) receptors was lower than that observed after 4 h carbachol treatment (55%, 59% and 35%, respectively) (see Tables 1 and 3). The comparison of [^{3}H]NMS and [^{3}H]QNB binding in unstimulated cells were comparable for M_1-M_5 subtypes suggesting that in untreated conditions, most of the muscarinic receptors are expressed on the cell surface. This is consistent with microscopic studies performed in striatal

dendrites using immunogold labeling technique where most of the muscarinic receptors (about 89%) were localized to the plasma membrane (Bernard et al., 1999) as well as binding studies performed in PC12 cells (Volpicelli et al 2002).

4.2 Characterization of the effect of proteasomal and lysosomal inhibitors on the downregulation of muscarinic receptors

In this investigation, we observed subtype-specific differences in the mechanisms of downregulation. The downregulation of $G_{q/11}$ coupled receptors (M_1, M_3 and M_5) was sensitive to proteasomal inhibitors. On the other hand, the downregulation of $G_{i/o}$ coupled M_2 receptors was inhibited by lysosomal inhibitor NH_4Cl. However, the carbachol-stimulated downregulation of M_4 receptors, which couple with $G_{i/o}$ proteins was sensitive to both proteasomal and lysosomal inhibitors. Hence, the differences in the mechanisms of downregulation in the muscarinic receptors were unrelated to the type of G protein they coupled with. Previous findings performed in histamine H_1 receptors a G_q coupled receptor revealed that the downregulation was inhibited by proteasomal inhibitors lactacystin and MG-132 (Hishinuma et al., 2010). The downregulation of G_i coupled κ, δ and μ opioid receptors were also sensitive to proteasomal inhibitors and not to lysosomal inhibitors (Li et al., 2000; Chaturvedi et al., 2001). This is in agreement with our study that the pathway of downregulation was independent of the type of G protein coupled.

Comparisons of the time-course of internalization of muscarinic receptors to the time-course of downregulation of muscarinic receptors (Figures 1 and 6) suggest that internalization is a pre-requisite for downregulation of muscarinic receptors (Li et al., 2000). The potencies of carbachol to elicit downregulation of M_2 and M_4 receptors (Figure 7) were also similar to the potencies of carbachol in causing internalization of these receptors (Tsuga et al., 1998b). This clearly suggests that internalization is a preceding step in downregulation of M_2 and M_4 receptors. In our time-course downregulation assays, we observed transient up-regulation of M_1 and M_3 receptors after 1 h of carbachol treatment following which downregulation continues. This could

be the result of a finite amount of receptor delivery to QNB accessible vesicle from QNB insensitive storage vesicle (Volpicelli et al 2002).

We observed that pretreatment of CHO cells expressing muscarinic receptors with proteasomal inhibitors in the absence of carbachol increased the expression of muscarinic receptors (Figure 12 and Table 4). In a typical cell, both constitutive degradation and biosynthesis of receptors takes place simultaneously. Proteasomal inhibitors might have inhibited the constitutive degradation of muscarinic receptors with no effect on the biosynthesis of receptors. Hence there is an increase in receptor expression in cells treated with proteasomal inhibitors alone when compared to vehicle treated cells. Our findings are consistent with the increased expression of δ and μ opioid receptors in HEK293 cells pretreated with proteasomal inhibitors alone (Chaturvedi et al., 2001).

M_1, M_3 and M_5 receptor downregulation was affected by proteasomal inhibitors only, while M_2 receptor downregulation was inhibited by lysosomal inhibitors only. M_4 receptor downregulation was affected by both proteasomal and lysosomal inhibitors. This is consistent with previous studies where downregulation of some GPCRs are affected by only proteasomal inhibitors (Chaturvedi et al., 2001; Hishinuma et al., 2010) while downregulation of few GPCRs are inhibited by lysosomal inhibitors only (Marchese and Benovic, 2001). Yet there are a few receptors whose downregulation was inhibited by both proteasomal and lysosomal inhibitors (Li et al., 2000; Dupre et al., 2003). The lysosomal inhibitor E-64-d did not have any significant effect on the downregulation of any of the muscarinic receptor. This might be the result of lysosomal enzymes inhibited by E-64-d not taking an active part in degradation of muscarinic receptors. Although there are cell-specific differences in the extent of downregulation of muscarinic receptors, the mechanisms of downregulation could be similar (Lenz et al., 1994). For example, the finding that M_4 receptor downregulation in CHO cells is affected by lysosomal inhibitor NH_4Cl is congruent with the reduction of muscarinic receptor downregulation by NH_4Cl in NG-108 cells which expresses M_4 receptors (Ray and Berman, 1989). No significant effect of

the lysosomal inhibitor NH_4Cl on the downregulation of M_3 receptors expressed in CHO cells is also consistent with no effect on the downregulation by NH_4Cl in vas deferens (Hiroshi et al., 1982), which expresses M_3 receptors (Silva et al., 1988).

Proteasomal inhibitors (PSI, MG-132 and lactacystin) significantly increased the downregulation of M_2 receptors. This is in sharp contrast to the inhibition of M_2 receptor downregulation in MEF cells by proteasomal inhibitor lactacystin (Mosser et al., 2008). In MDCK cells, Shmuel et al (2007) have shown that only a very small fraction of M_1 receptors colocalize with Rab7 after agonist-stimulation suggesting that there could be pathways alternate to the lysosomal pathway for the degradation of M_1 receptors. This is consistent with the current study where no significant effect of lysosomal inhibitors on the downregulation of M_1 receptors was observed. Proteasomal inhibitor PSI did not have any inhibitory effect on the downregulation of M_2 receptors at both shorter and longer incubation times with carbachol. Similarly lysosomal inhibitor NH_4Cl did not have any inhibitory effect on the downregulation of M_1 receptors.

All of the above results point to a mechanism where the degradation of GPCRs happens (lysosomes only, proteasomes only or combination of both). In the first pathway, the degradation happens completely in the lysosomes or proteasomes. An example for degradation happening completely in lysosomes is agonist-induced downregulation of CXCR4 receptors. The downregulation of histamine H_1 receptors is completely inhibited by proteasomal inhibitors. In the second pathway, one fraction of the receptors is degraded in the lysosomes, while the other in the proteasomes. A very good example of this pathway is the agonist-induced downregulation of PAFR where the inhibition of proteasomal and lysosomal inhibitors is additive. There is a third possibility that the downregulation proceeds initially in the proteasomes where the receptor might be digested partially to a radioligand unidentifiable fragment. This protein fragment is then transported to the lysosomes where final degradation takes place. This study is the first of its kind to study the mechanism of downregulation of muscarinic receptors in a heterologous cell line with a battery of proteasomal and lysosomal inhibitors. Proteasomal and lysosomal inhibitors

differentially inhibited the downregulation of muscarinic receptors. These differences could be due to the variations in sequences between muscarinic receptors. Sequence variations in muscarinic receptors are prominent in the third intracellular loop and C-terminal tail region when compared to other regions (Hulme et al., 1990). As a result of sequence variations in the cytoplasmic regions (i3 loop and C-terminal tail) involved in trafficking, there could be differences in the ubiquitination patterns between subtypes. Polyubiquitination and monoubiquitination serve to downregulate proteins in proteasomes and lysosomes, respectively. The amino acid sequence of M_1, M_3 and M_5 receptors are similar and have significant variation compared to M_2 and M_4 receptors, which have similar sequence (Hulme et al., 1990). Consistent with the sequence homology, M_1, M_3 and M_5 receptor downregulation is inhibited by proteasomal inhibitors while M_2 and M_4 receptor downregulation is inhibited by lysosomal inhibitors.

4.3 Characterization of the role of the third intracellular loop and C terminal tail of M_1receptors

We wanted to determine domains in M_1 receptor responsible for agonist-induced downregulation. Since internalization is required for downregulation, we wanted to make deletion mutations without affecting agonist-induced internalization. Lameh and Philip (1992) made several deletion mutants in the third intracellular loop region and C-terminal tail region of M_1 receptors in pursuit for identifying domains responsible for agonist-induced internalization. Out of the several mutations made by them, we selected two deletion mutants which internalized and signaled to similar extents as wild-type M_1 receptors. One of the mutant had a domain in the i3 loop deleted (M_1 del 276-282) while the other mutant had a deletion in the C-terminal tail (M_1 del 447-459). In this investigation, we created the two deletion mutant versions of M_1 receptors (M_1 del 276-282 and M_1 del 447-459) using site directed mutagenesis and expressed stably in CHO cells. The affinities of the mutant M_1 receptors for [^{3}H]NMS were comparable to that of wild-type M_1 receptors. However, the potency (EC_{50}) of mutant receptors was at least 3-fold greater than

that of wild-type M_1 receptors. The maximum response (E_{max}) of the mutant receptors in response to carbachol was considerably different from the wild-type receptor. M_1 del 276-282 receptors expressed lesser number of receptors (see Table 8) and signaled 1.5 times greater than the wild-type receptor (see Table 7 and Figure 11). Since agonist-dependent signaling is enhanced in M_1 del 276-282 receptors, desensitization might be affected. This is in agreement with previous studies performed by Jewell-Motz and Ligget (1995) in CHO cells expressing α_2C2 adrenergic receptors. They identified a string of acidic amino acids in the i3 loop of $\alpha 2C_2$ adrenergic receptor to be involved in desensitization and agonist-induced phosphorylation. Deletion mutants lacking this acidic domain internalized to the same extent as wild-type receptors and bound to antagonist with a comparable affinity as wild-type receptors. Studies performed using peptide substrates have identified that a stretch of acidic amino acids N terminal to the Ser, Thr phosphorylation sites serve as the GRK2 binding domains on the receptor (Chen et al. 1993; Onorato et al. 2002).The consensus sequences of phosphorylation of various second messenger dependent kinases such as PKA and PKC have also been identified. None of these consensus sequence match the acidic sequence, we deleted (Kennelly and Krebs 1991). The consensus sequence of casein kinase 1 binding domain is a stretch of acidic amino acids N terminal to the phosphoacceptor serine/threonine (Kennelly and Krebs 1991). M_1 receptors have been known to be phosphorylated in an agonist-dependent manner by GRK2 and casein kinase 1(Haga et al 1996; Waugh et al 1999). Hence, the domain deleted by us in the i3 loop might be a binding site for GRK or casein kinase 1 involved in agonist-induced phosphorylation of M_1 receptors.

M_1 del 447-459 receptors expressed nearly same amount of receptor as the wild-type receptor (see Table 8) but signaled only half the amount as wild-type receptor (see Table 7 and Figure 11). Deletion of residues in the C-terminal tail region of M_1 receptors decreased the potency of M_1 receptors. The amount of signaling was also reduced. This could have been the result of a conformational change introduced by the deletion mutation. The conformational

change could have also caused decreased G protein-coupling as it is known that G protein coupling requires amino acids in the cytoplasmic regions of GPCRs (Teller et al., 2001).

In this study, the rate and extent of M_1 wild-type, M_1 del 276-282 and M_1 del 447-459 receptor internalization, recycling and downregulation in CHO cells were compared. There are no significant differences in the rates and plateaus of internalization between the wild type and mutant M_1 receptors. In conclusion, the deletion mutations introduced in the third intracellular loop region and C terminal region of M_1 receptor had no effect on either the rate or extent of internalization.

We observed a significant difference in the initial rates and extent of delivery of M_1 wild-type receptors in the cells treated with carbachol and untreated cells indicating that wild-type receptors recycled. The initial rate of delivery of M_1 wild-type receptors in carbachol treated cells was 2.5 fold greater than the rate in untreated cells. The extent of recovery was significantly greater in treated cells and was 1.4 fold greater than in untreated cells.

In cells expressing M_1 del 276-282 receptors, there are no differences in the rate of recovery in carbachol treated and untreated cells. The extent of recovery of muscarinic M_1 del 276-282 receptors in carbachol treated cells were not significantly different than the extent of recovery in untreated cells. In conclusion, deletion of amino acids 276-282 impaired recycling of muscarinic M_1 receptors.

In cells expressing M_1 del 447-459 receptors, there are no differences in the rate of recovery in carbachol treated and untreated cells. The extent of recovery of muscarinic M_1 del 447-459 receptors in carbachol treated cells were not significantly different than the extent of recovery in untreated cells.

The basal plasma membrane delivery of M_1 del 447-459 receptors was significantly higher than the basal plasma membrane delivery of M_1 wild type receptors in both the rate and extent of delivery. This suggests that deletion of amino acids 447-459 in M_1 receptors accelerates their delivery to the plasma membrane.

The downregulation of M_1 receptors in response to prolonged treatment with carbachol was significantly reduced in the cells expressing either of the deletion mutants studied. This suggests that the deleted regions play a role in mediating carbachol-induced downregulation. The C terminal tail deletion region (K447-Q459) did not downregulate in response to carbachol (Figure 14 and Table 10). Even though internalization remains indifferent between the mutants and wild-type receptor until 4 h of carbachol treatment, where a plateau is attained, the extent of internalization was significantly different between wild-type and mutant receptors at 24 h of carbachol treatment. While internalization of wild-type receptors continues after reaching a plateau, the internalization of M_1 del 276-282 receptors retains the same plateau even after 24 h of carbachol treatment. The internalization of M_1 del 447-459 receptors reached a plateau at 4 h which is similar to the plateau reached by M_1 wild-type or M_1 del 276-282 receptor at the same time period. At 24 h of carbachol treatment, the internalization of M_1 del 447-459 receptors decreased. This could be due to increased delivery of receptors to the plasma membrane from the biosynthetic pathway. There are no differences in the [^{3}H]NMS and [^{3}H]QNB binding in unstimulated cells for M_1 wild-type and M_1 del 276-282 receptors suggesting that most of the receptors reside in the plasma membrane (Figure 14 and Table 10). In contrast, a significant amount of expressed M_1 del 447-459 receptors remain intracellularly as evident from the [^{3}H]NMS and [^{3}H]QNB binding in unstimulated cells (Figure 14 and Table 10).

4.4 Summary and future directions

This dissertation represents the comparison of internalization, recycling and downregulation of muscarinic receptors expressed in CHO cells. For the first time, the comparison of effect of proteasomal inhibitors and lysosomal inhibitors on the downregulation of muscarinic receptors expressed in CHO cells was studied. Additionally, a domain in the C terminal tail of M_1 receptors has been identified to be involved in downregulation of M_1 receptors.

Carbachol treatment caused internalization, recycling and downregulation of muscarinic receptors in CHO cells. Subtype-specific differences in the rate or extent or both of

internalization, downregulation and recycling of muscarinic receptors were identified. Based on these findings, it would be interesting to determine whether there are subtype-specific differences in the G protein-coupled receptor kinases and arrestins that interact with the receptors during agonist treatment. The type of G protein-coupled receptor kinase (e.g., GRK 2, 3, 5 and 6) and arrestin (e.g., arrestin 2 and 3) recruited to a particular muscarinic receptor subtype may be responsible for the subtype-specific differences observed in this study.

Proteasomal inhibitors and lysosomal inhibitors differentially inhibited the downregulation of muscarinic receptors. While proteasomal inhibitors inhibited the downregulation of M_1, M_3, M_4 and M_5 receptors, they enhanced the downregulation of M_2 receptors. The lysosomal inhibitors significantly decreased the downregulation of M_2 and M_4 receptors. In summary, there are subtype-specific differences in the mechanisms of carbachol-induced downregulation of muscarinic receptors. Based on the results obtained, it is clear that downregulation of M_1, M_3 and M_5 receptors may happen in the proteasomes. Since ubiquitination is a prerequisite for downregulation in the proteasomes, it would be interesting to determine whether there are subtype-specific differences in the pattern and number of ubiquitination. Mono-ubiquitination of proteins have been thought to be a targeting signal for proteins destined for lysosomes, while poly-ubiquitinated proteins are known to be degraded in the proteasomes. There are seven lysine residues within the ubiquitin molecule. The trafficking of polyubiquitinated proteins depends on the lysine residue involved in chain formation. The lysine K48 ubiquitination has been shown to be involved in proteasomal degradation, while K63 lysine ubiquitination has been shown to be involved in endocytosis and DNA repair (Pickart, 2000). It would also be interesting to determine the extraction of GPCRs by proteasomal machinery.

Deletion mutants were created in the i3 loop and C-terminal tail region of M_1 receptors. Both the mutants created were functional and were able to bind to the muscarinic receptor antagonist NMS. The deletion mutants have an internalization profile similar to that of wild-type receptor. The downregulation was affected by both deletion mutants. The greatest effect on

downregulation was seen with the deletion of K447-Q459 amino acids in the C terminal tail. Analysis of this domain revealed the possession of a class I SH3 domain ligand and three hydroxyl amino acids (Li, 2005). Future studies should be directed towards identifying the mechanism by which this domain regulates agonist-induced downregulation of M_1 receptors.

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VITA

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Doctor of Philosophy

Thesis: CHARACTERIZATION OF INTERNALIZATION, RECYCLING AND DOWNREGULATION OF MUSCARINIC RECEPTORS

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Major Field: Biomedical Sciences

Scope and Method of Study: Muscarinic receptors mediate the responses elicited to the activation of the parasympathetic nervous system. Specifically, muscarinic receptors mediate exocrine glandular secretion, smooth muscle contraction and decreased heart rate and contractile force. Five subtypes of muscarinic receptors have been cloned and they undergo a process of agonist-dependent regulation, which involves desensitization, internalization, recycling and downregulation. This study was designed to characterize the kinetics and extent of internalization, recycling and downregulation of muscarinic receptors expressed individually in CHO cells and to identify subtype-specific differences in these processes. The mechanisms of downregulation of muscarinic receptors expressed in CHO cells were also investigated by using proteasomal and lysosomal inhibitors. Additionally, deletion mutations were made in the third intracellular loop and C-terminal tail region of the muscarinic M_1 receptor to identify regions responsible for recycling or downregulation.

Findings and Conclusions: There are subtype-specific differences in the internalization, recycling and downregulation of muscarinic receptors. The rank order for carbachol-induced internalization was $M_2 > M_4 = M_5 > M_3 = M_1$. Unlike M_2 receptors, M_1, M_3, M_4 and M_5 receptors recycled back to the plasma membrane following 1 h carbachol treatment. M_1 receptor downregulated to a greater extent compared to other subtypes. The downregulation of M_1, M_3, M_4 and M_5 receptors was affected by proteasomal inhibitors, while lysosomal inhibitors affected the downregulation of M_2 and M_4 receptors. The M_1 deletion mutants (M_1 del 276-282 and M_1 del 447-459) signaled through activation of phospholipase C activation and were able to bind to [^{3}H]NMS. Additionally, the internalization of M_1 deletion mutants was indifferent from those of the wild-type M_1 receptor. However, both deletion mutants had an impaired recycling and downregulation. The C-terminal deletion (K447-Q459) significantly affected the downregulation of M_1 receptor, suggesting a role of this domain in mediating M_1 receptor downregulation.

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