EFFECT OF CHLORPYRIFOS OXON ON M2 MUSCARINIC ACETYLCHOLINE RECEPTOR REGULATION

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1999

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2004

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ACKNOWLEDGMENTS

My sincerest gratitude goes to my major advisor, Dr. Carey N. Pope for the intelligent supervision, for providing inspiration to do this work.

I am also thankful to my committee members, Dr. Cyril Clarke, Dr. Charlotte Ownby and Dr. Doris Patneau for helpful comments on the content and form of this manuscript.

I am indebted to the Fulbright-Philippine Agriculture Scholarship Program (FPASP) and the Philippine American Education Foundation (PAEF) whose exchange program deepened my understanding of the U.S. culture and its people and allowed me to promote mutual understanding between the U.S. and the Philippines.

I am grateful to the University of the Philippines in Mindanao (UPMINDANAO) for supporting my pursuit for graduate studies, the National Institute of Environmental Health Sciences (NIEHS), Oklahoma State University Board of Regents and Dr. Sidney Ewing, Wendell H. and Nellie G. Krull Endowed professor for the financial assistance.

I am also thankful to the following: Ms. Sharon Baker for doing the preliminary work on the project; Dr. Liming Zou for vital inputs on M2 receptor regulation; Dr. Subramanya Karanth, Dr. Jing Liu Pope and Dr. Shaikh for technical and statistical help; Dr. Greg Sawyer, Ms. Tiffany Ricks and Mr. Jon Hart for technical assistance in cell culture techniques; Dr. Tom Bonner and Dr. Jelveh Lameh for providing the CHO cells used in the study, Dr. Laura A. Volpicelli for help with the immunocytochemistry and imaging techniques; Dr. Telugu Narasa Raju and Dr. Reddy for help with the fluorescent microscope; Ms. Betty Handlin for document formatting.

My deepest thanks to Ms. Paetra Hauck (IIE), Dr. Adel Tongco, Randy Beckloff and Amy Fair (OSU-ISS) for administrative help during my stay here at OSU; the Filipino community here in Stillwater for being my family away from home; to my roommate Brenda Tubaña for the statistical analysis and camaraderie and all my friends who have helped me survive here in the U.S. just by being there.

I want to extend my appreciation also to my greatest friend and husband, **Dr. Rex Rhoderick Zamora**, for making my adjustment in the US easier and especially for the love, patience and understanding all throughout the conduct of this work.

I am also thankful to my family in the Philippines, my mom Elsie, my dad Marvelino and my siblings Leif Marvin and Marielle who continue to inspire me; and to the Zamoras, Dr. Emilio, Ampy and Zandra, for their love and support.

Most of all, I am forever grateful to God, my Lord and Saviour, for the gift of life that allowed me to experience all the wonderful things He has created. And most especially for the graces He has bestowed on me. I am nothing without Him.

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INTRODUCTION

Background and Significance

Humans have always been in constant battle with undesirable plants and animals that threaten the food supply and health. The first use of chemicals to control insect and pests can be traced to the time of Homer at around 1000 B.C. where sulfur was burned as a fumigant. Pliny the Elder (A.D. 760) recorded in his *Natural History* the use of the gall of green lizard to protect apples from rotting and from worm infestation. In the late 1600s, extracts from plants like tobacco (*Nicotiana tobacum),* the seed of nux vomica (*Strychnos nuxvomica),* pyrethrum flowers (*Chrysanthemum cinerariaefolium)* and *Derris elliptica* were used to control insects or kill rodents. Different forms of sulfur and arsenic were extensively used in agriculture in the 1900s. Insecticides and other pesticides have afforded humans a more abundant supply of food and protection from infectious diseases. However, along with the beneficial effects brought by these agents, considerable risks are inherently associated with their use(Klaassen 1996).

Insecticides and Pesticides

Most of the insecticides used today are targeted to the insects' nervous system. The nervous system in insects is highly developed and has some similarities with that of the mammalian nervous system. The structure of chemicals used in the development of insecticides was transformed to fit a specific molecule involved in physiological, cellular, biochemical or molecular processes within the nervous system. Since insecticides tend to be non-selective, the nervous system of non-target species can often be affected leading to toxicity to non-target species(Klaassen 1996). The mechanisms by which insecticides elicit

neurotoxicity can be generally classified into three groups: ion transport disruption, enzyme inhibition and neurotransmitter release alteration.

Organochlorines

The first major group of organic insecticides synthesized was the organochlorines(Klaassen 1996). Paul Muller, a Swiss entomologist, was awarded the Nobel Prize in 1948 for his discovery of the biocidal properties of DDT (dichlorodiphenyltrichloroethane), a prototype organochlorine insecticide that effectively controlled a variety of household, crop and public health pests. DDT was extensively utilized during World War II to control malaria, yellow fever and other arthropod-borne diseases afflicting the troops(Brooks 1974). Since that time, a number of other effective insecticidal organochlorines including aldrin, dieldrin, chlordane and benzene hexachloride have been synthesized(Chambers 1992).

Organochlorines have low water solubility, are chemically stable and have been used for a variety of purposes in agriculture, forestry, residential and commercial structures to control pests. During application, other non-target species can be affected and because organochlorines persist in the environment and are not easily degraded, there is a potential for bioconcentration and biomagnification within food chains. As first brought to mass attention by Rachel Carson in the historic monograph *Silent Spring*, wildlife species were found to accumulate biologically active organochlorines(Carson 1962). Organochlorines such as DDT were found to substantially hamper reproductive performance and fertility of wildlife species, in particular those at the top of food chains as residues accumulated across species(Stickel 1968; McFarland and Lacy 1969; Longcore, Samson *et al.* 1971; McBlain, Lewin *et al.* 1974; Crum, Bursian *et al.* 1993). A monitoring program conducted in 1994, 1997, 1998, 1999, and

2001 in the Asia-Pacific region (Cambodia, China, Hong Kong, India, Indonesia, Japan, Korea, Malaysia, Philippines, Far East Russia, Singapore, and Vietnam), reported that organochlorines like DDT and hexachlorocyclohexane residues were found in mussels in all the areas sampled (Monirith, Ueno *et al.* 2003).

Organophosphates

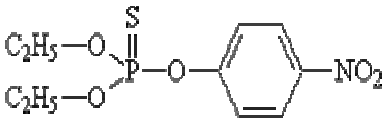
Organophosphates (OPs) are a large class of synthetic compounds with a wide variety of uses. Unlike the organochlorines, OPs are relatively unstable, do not persist in the environment, and therefore pose less danger of biomagnification. The insecticidal action of OPs was first discovered in Germany during World War II, in search for a substitute for the insecticide nicotine, which was in short supply at that time. The German chemist Gerhardt Schrader, known as the "Father of OP insecticides", synthesized several organophosphate insecticides including dimefox, schradan and parathion and the nerve agents tabun and sarin (Chambers 1992).

Classification

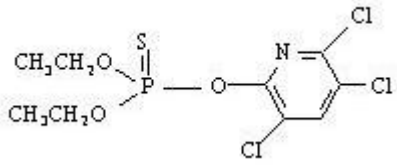
"Organophosphates" (OPs) is a common name referring to all organophosphorus compounds. Most of the OPs are derived from chemical reactions of phosphoric acid, dating from 1820 when Lasaigne first synthesized triethyl phosphate to the present large variety of synthetic derivatives of phosphoric acid (Chambers 1992) . All OPs are esters of phosphoric or thiophosphoric acid with various substitutions of oxygen, sulfur, nitrogen, and carbon resulting in six different subclasses: "true" phosphates, phosphonates, phosphorothionates, phosphorodithioates, phosphorothiolates and phosphoramides(Chambers 1992). The most commonly used OPs have four atoms attached directly to the phosphorous (P) atom. For the

"true" phosphates, four atoms of oxygen surround the P atom, resulting in high reactivity and generally rapid chemical action(Chambers 1992).

Chlorpyrifos (CPF), parathion (PT) and methyl parathion (MPT) are examples of phosphorothionates having sulfur in their structures, i.e., derivatives of thiophosphoric acid(Chambers 1992). Chlorpyrifos and parathion are structural analogs that differ in their leaving groups. The leaving groups of chlorpyrifos and parathion are 3,5,6 trichloro-2-pyridinol (TCP) and p-nitrophenol, respectively. The leaving group is that portion of the OP molecule that is released and excreted after phosphorylation of a macromolecule, as will be described in later.



Parathion



Chlorpyrifos

Figure 1. Chemical structures of parathion and chlorpyrifos.
Parathion and chlorpyrifos are structural analogs which differ in their leaving groups. Both belong to the same class, phosphorothionates(Chambers 1992).

Uses of OPs

OPs are widely used as pesticides. According to the US Environment Protection Agency (USEPA)(http://www.epa.gov/pesticides/op/primer.htm), almost 60 million pounds of OPs were used annually on almost 60 million acres of US agricultural crops including corn, cotton, canola, alfalfa, fruits, nuts and vegetables. Non-agricultural uses accounted for 17 million pounds of OPs and included treatment of livestock and pets, treatment of residential and commercial establishments against termites, fleas, cockroaches, ants and other household

pests and the treatment of ornamental lawns and turfs. OPs are cheap and have broad-spectrum activity. Because of the wide use of the pesticides in both agricultural and residential settings, people can be exposed on a regular basis through aggregate routes of exposure.

OPs have also been used as nerve agents, both in chemical warfare and chemical terrorism. During the early synthesis of OP insecticides in the 1940s, some OPs proved to be extremely potent neurotoxicants. Schrader synthesized two potent nerve agents, tabun and sarin, during the course of insecticide development(Chambers 1992). Other nerve agents synthesized later include soman and VX. Chemical warfare agents posed a threat during World War II and are still of concern today. However, the OP pesticides in use today are far less potent AChE inhibitors than the fast-acting nerve agents and typically require bioactivation to be effective(Chambers and Oppenheimer 2004). Most OPs require cytochrome P-450-mediated activation *via* oxidative desulfuration which leads to the production of "oxons", the active metabolites of OPs(Butler and Murray 1997).

Most OPs are classified as anticholinesterases (cholinesterase inhibitors). Some have been used or evaluated for the management of clinical diseases including Alzheimer's disease (AD), Parkinson's disease (PD), myasthenia gravis and glaucoma. AD and PD are characterized by diminished cholinergic neurotransmission(Knopman 1998). The auto-immune disorder myasthenia gravis is characterized by antibodies against acetylcholine receptors. Anticholinesterases cause accumulation of ACh allowing more interaction of signal molecules (acetylcholine) with cholinergic receptors and thereby promoting cholinergic neurotransmission. If a disease involves reduced cholinergic neurotransmission, inhibition of acetylcholinesterase can provide a therapeutic benefit.

The loss of neurotransmission in Alzheimer's disease (AD) has led to the therapeutic use of anticholinesterases for this disorder. Inhibition of acetylcholinesterase leads to an increase in available acetylcholine, shown to improve cognition in AD patients(Wilkinson, Francis *et al.* 2004). To date, the only FDA-approved drugs for the management of AD are anticholinesterases(Van Dyck 2004). Parkinson's disease is characterized by the degeneration of dopaminergic receptors in the brain(Wichmann and DeLong 2003). Anticholinesterases are also part of the management of Parkinson's disease because decreased dopamine results to an increase in acetylcholine leading to cholinergic toxicity. Management of Parkinson's disease includes administration of L-dopamine, dopaminergic agonists and anticholinesterases. Clinical trials in humans have shown that anticholinesterases improve cognitive functions(Giladi, Shabtai *et al.* 2003).

Mechanism of action

OPs interfere with degradation of the neurotransmitter acetylcholine by phosphorylating and blocking the action of the enzyme acetylcholinesterase (AChE). Normally, AChE efficiently hydrolyzes ACh into choline and acetate. During catalysis, AChE is acetylated (acetyl-AChE) at its catalytic center and choline is subsequently released. Hydrolysis of the acetyl-AChE complex liberates the free enzyme. OPs mimic ACh and interact with the enzyme in an analogous manner as ACh. Instead of being acetylated, however, AChE is organophosphorylated at its active site serine, forming a far more stable phosphoryl-AChE. Hydrolysis of the phosphoryl-AChE is very slow (hours) as compared to hydrolysis of acetyl-AChE (e.g. 0.15 msec)(Chambers 1992).

As a result of AChE inhibition, OP toxicity leads to the accumulation of ACh in synapses of the central and peripheral nervous systems. Excessive stimulation of post-synaptic

cholinergic receptors due to ACh accumulation subsequently follows. Finally, changes in post-synaptic cellular function due to the excessive stimulation of cholinergic receptors leads to functional signs of cholinergic toxicity. Modulation of any of these steps could theoretically influence the expression of cholinergic toxicity(Pope 1999).

Chlorpyrifos

The rate of phosphorylation and hence inhibition of AChE depends on the unique structure of the particular OP. OPs which have leaving groups that are electron donors tend to have lesser AChE inhibitory potency. The oxons of chlorpyrifos (which contains halogens), and parathion (which contains a nitro leaving group) are relatively similar in potency *in vitro*. Compared to parathion, however, chlorpyrifos is a markedly less potent AChE inhibitor *in vivo*. Both CPF and parathion must be biotransformed into chlorpyrifos oxon and paraoxon through oxidative desulfuration by cytochrome P450 in the liver to elicit toxicity (Figure 2)(Chambers 1992; Dai, Tang *et al.* 2001). The differential potencies *in vivo* relate to markedly different detoxification capacities.

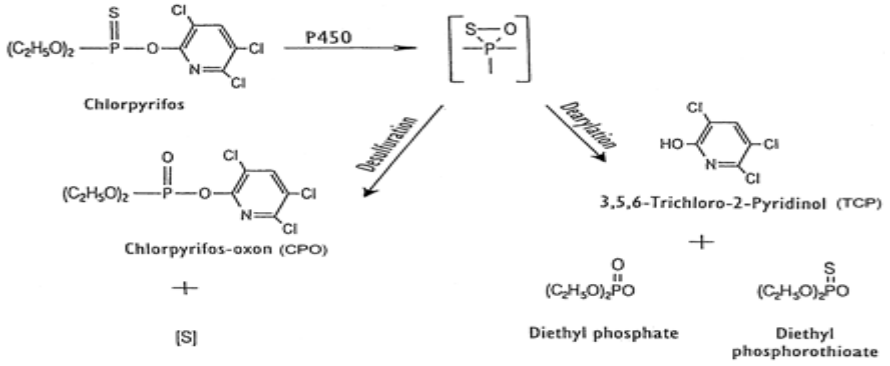


Figure 2. Oxidative desulfuration of chlorpyrifos.
Chlorpyrifos is biotransformed via oxidative desulfuration into its active metabolite, chlorpyrifos oxon (CPO) by cytochrome P450 in the liver(Dai *et al.* 2001).

Chlorpyrifos (e.g. Dursban®, Lursban®) is a widely used OP insecticide in the U.S. and throughout the rest of the world(NASS 2003). Chlorpyrifos exists in a variety of preparations including wettable powder, bait, emulsifiable concentrate, dust, pellets, pressurized liquid, microencapsules, impregnated material, etc.(Chambers 1992; Dai *et al.* 2001). It was first registered for use in the US in 1965 and has been extensively used to control pests in soil and foliage during crop production. In 2002, the US Department of Agriculture (USDA) reported use of a total of approximately 1.5 million lbs of chlorpyrifos in corn production in the states of Illinois, Indiana, Iowa, Nebraska, Ohio and Wisconsin alone(NASS 2003). Approximately 800 products containing chlorpyrifos were registered for household, agricultural (e.g. corn, peanuts, alfalfa) and veterinary purposes (flea collars, ear tags for cattle)(Smegal 2000).

Food Quality Protection Act of 1996

The wide-spread use of insecticides and pesticides over the last 50 years has increased concern for the overall health effects of these chemicals in humans and other non-target species. Evidence of widespread contamination by chlorpyrifos has been reported. Several studies found chlorpyrifos metabolites in the urine of children in both OP-sprayed agricultural regions and urban environments(Wessels, Barr *et al.* 2003). This is of particular concern because a number of experimental studies have also reported that chlorpyrifos may be more toxic in young animals (Pope 1999; Moser 2000; Qiao, Seidler *et al.* 2002; Betancourt and Carr 2004).

In a review by Loevinsohn and Rola, the use of pesticides in the Philippines increased dramatically in the 1970's during the Green Revolution. The Fertilizer and Pesticide Authority (FPA) was created in 1977 by the Philippine government. It was not until 1980's and early

1990's before policy decisions on regulation of use of pesticides were made. Part of the policy on pesticide use was the restriction of the use of chemicals classified by the World Health Organization as category 1 chemicals. In the 1980's, the Philippine FPA banned pesticides including parathion ethyl, DDT among others (Loevinsohn 1998).

Over the years, several US laws have been passed to protect consumers from toxic residues in food. The Federal Food, Drug and Cosmetics Act (FFDCA), which replaced the first Food and Drugs Act (FDA), was passed in 1938. Later on, the FFDCA was amended by the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), which was passed by US Congress in 1947(Klaassen 1996). The Food Quality Protection Act (FQPA) was signed into law on August 3, 1996 by President Bill Clinton. FQPA is an amendment of the FFDCA and FIFRA (http://www.epa.gov/opppsps1/fqpa/ 2004) and was meant to unify these 2 laws and establish science-based assessment of pesticide use in the US. Like the FFDCA and FIFRA, the overall goal of FQPA is to ensure that US consumers have access to a safe and abundant food supply. The FQPA mandates a single, health-based standard for all pesticides in all foods(http://www.epa.gov/opppsps1/fqpa/ 2004). The FQPA is administered by the United States Environmental Protection Agency (US EPA) which is mandated by law to formulate registration requirements and conduct appropriate tests to establish pesticide tolerance levels for residues in raw and processed foods, to conduct chemical and toxicological evaluations, perform environmental impact assessments, and provide labeling and use specifications and restrictions(Klaassen 1996).

An important concept in FQPA is "common mechanism" of toxicity. EPA is mandated under FQPA to establish tolerance levels for all pesticides that elicit toxicity through a common mechanism. To determine the mechanism of toxicity of a pesticide, "the major

steps leading to adverse health effects following interaction of a pesticide with biological targets" should be known. Therefore, describing the cascade of events after interaction with biological targets and the effects following this interaction is crucial in determining the mechanism of toxicity of a pesticide. Pesticides are determined to share a "*common mechanism of toxicity*" if they cause the same toxic effects in or at the same organ or tissue by the same sequence of major biochemical events as supported by scientifically reliable data. Of the three groups of pesticides that are currently subject to EPA tolerance evaluation based on common mechanism of toxicity, OPs were the first to undergo cumulative risk assessment(Pope 1999).

Cumulative risk assessment and common mechanism of toxicity

Cumulative risk assessment of pesticides working through a common mechanism evaluates the **combined** toxicity of different pesticides. For OPs, the common mechanism of toxicity evaluated in risk assessment is initiated by acetylcholinesterase inhibition. Data are evaluated for each pesticide with both acute and chronic endpoints and then the relative impacts of different OPs occurring under different use conditions are estimated. Through this process, the relative effectiveness of different OPs in inhibition of acetylcholinesterase can be estimated and tolerance levels and exposures then controlled to minimize cumulative toxicity.

In June 2000, the EPA reported the results of the re-assessment of chlorpyrifos as mandated by FQPA. EPA reported that regulations controlling chlorpyrifos use did not provide adequate protection for children and initiated several steps to address this issue. EPA issued restrictions on use of chlorpyrifos in residential areas, except by professional applicators, prohibited its use in parks and schools where children may be present, decreased chlorpyrifos tolerance levels for apples and grapes and prohibited its use and repealed the tolerance levels for chlorpyrifos in tomatoes. Whereas household use of chlorpyrifos was

restricted after 2001, it was still extensively used in the production of corn and other agricultural products (NASS 2003) posing continued risk of pesticide exposure from food. In June 2002, two years after EPA released its first reassessment of chlorpyrifos, EPA released the Revised OP Cumulative Risk Assessment. The cumulative risk assessment reported that tolerances were consistent with the highest levels of safety for over 1,000 OP products that were evaluated. Chlorpyrifos was reported not to have age-dependent sensitivity, which allowed the removal of the additional FQPA safety factor for chlorpyrifos intended to protect children. This implies that chlorpyrifos does not pose additional health risks to children(http://www.epa.gov/oppsrrd1/op/chlorpyrifos/consumerqs.htm 2004). Considering the number of publications indicating potential neurodevelopmental effects of chlorpyrifos, this is a debatable conclusion.

In the 2002 cumulative risk assessment report, the only endpoint measured was the inhibition of brain AChE. Whereas the report described potential sources of OP exposure like drinking water and from residential applications, the adverse effects of OPs on the nervous system including motor activity, learning and memory, reflexes and sensory abilities and development were not included in the assessment.

As noted before, because OPs inhibit breakdown of ACh, leading to overstimulation of post-synaptic cholinergic receptors and consequent changes in cellular function, modulation at any point in this cascade could influence the outcome after OP exposure. While cumulative risk assessment for OPs assumes a common mechanism of toxicity, a number of studies suggest that some OPs have additional sites of action that might have toxicological relevance. Differential degrees of toxicity observed in some studies by OPs with similar effects on AChE activity might be explained by interaction with such other non-cholinesterase targets. If direct

interaction with other macromolecules modulate OP toxicity, these actions could impact the assessment of cumulative risk based on a common mechanism of action (Pope 1999) and refine pesticide regulatory decisions. Conversely, pesticide regulation based mainly on a common mechanism of toxicity does not fully consider other effects which may be relevant, especially with long-term and simultaneous OP exposures.

Non-cholinesterase targets

OPs have been found to have non-cholinesterase targets, some of which may have toxicological relevance at low levels of exposure(Pope 1999). For example, OPs have been reported to interact with other esterase enzymes. The prototype OP anticholinesterase diisopropyl phosphorofluoridate (DFP) and chlorpyrifos inhibited neuropathy target esterase (NTE), a specific carboxylesterase found in the nervous system(Clothier and Johnson 1980; Lotti and Moretto 1993). Carboxylesterases (CEs) are classified as B-esterases, i.e. they are sensitive to inhibition of some OP compounds(Mendoza, Shields *et al.* 1971; Murphy and Cheever 1972; Chambers and Carr 1993). Carboxylesterases stoichiometrically react with OPs preventing OP interaction with and inhibition of AChE. The CE-mediated interaction is believed to be particularly important in the detoxification of potent OPs like nerve agents. Inhibition of NTE due to exposure to acute high dosages of some OPs is believed to cause organophosphate-induced delayed neuropathy (OPDIN), a rare clinical condition generally elicited by ingestion of massive amounts of OPs. Inhibition of NTE in some way leads to degeneration of sensory and motor neurons of the peripheral nerves and the spinal cord(Richardson 1995). NTE (-/-) mice die in the during prenatal development whereas NTE (+/-) mice are hyperactive and more sensitive to OP toxicity than the wild type(Glynn 2003). OPIDN cases present with paresis in the lower limbs and motor axonal

neuropathy(Carrington and Abou-Donia 1988; Lotti, Caroldi *et al.* 1991; Vasconcellos, Leite *et al.* 2002).

Neuropathic OPs like phenyl saligenin phosphate (PSP) or mipafox caused morphological changes in cultures of chick dorsal root ganglion including a decrease in neurite length-to-diameter ratios, dissolution of neurofilaments and microtubules and degradation of mitochondria. Paraoxon (a non-neuropathic OP), however, did not cause such changes(Carrington and Abou-Donia 1988; Lotti *et al.* 1991; Vasconcellos *et al.* 2002).

Butyrylcholinesterase (BuChE) is an enzyme that can hydrolyze ACh although its physiological function is unknown(Wang, Schopfer *et al.* 2004). BuChE was found to be sensitive to the effects of some OPs. Acute exposure to parathion caused significant reduction in BuChE levels in the liver of three-spined stickleback (Gasterosteus aculeatus)(Wogram, Sturm *et al.* 2001).

Non-esterase enzymes were also found to be additional targets of OPs. The OP prototype DFP, used for decades in biochemistry laboratories to inhibit proteases, inhibited the digestive enzymes chymotrypsin and elastase, the blood clotting factor thrombin (Pruett, Chambers *et al.* 1994; Quistad and Casida 2000) and acylpeptide hydrolase(Richards, Johnson *et al.* 2000). .

Cholinergic Receptors as OP Targets

Acetylcholine (ACh) facilitates many a variety of actions in the central and peripheral nervous systems from muscle contraction to learning and memory via binding to and activating cholinergic receptors(Klaassen 1996). Past studies have shown that OPs can directly bind to target cholinergic receptors(Eldefrawi, Schweizer *et al.* 1988; Katz, Cortes *et al.* 1997).

There are two basic types of cholinergic receptors. Nicotinic receptors mediate "fast" cholinergic responses whereas G-protein coupled muscarinic receptors typically mediate modulatory responses in the brain and peripheral tissues(Caulfield and Birdsall 1998; Eglen 2001). Some OPs were shown to bind to nicotinic receptors in neuromuscular junctions(Eldefrawi and Eldefrawi 1983; Eldefrawi *et al.* 1988; Katz *et al.* 1997). The active metabolites of some OPs caused differential effects on the function of nicotinic autoreceptors on ACh release. In rat synaptosomes, paraoxon had no significant effect on nicotinic autoreceptor function (NAF) while chlorpyrifos oxon completely blocked NAF *in vitro(Wu, Sun et al. 2003)*. Chlorpyrifos exposure *in vivo* also inhibited NAF in rats of different age groups(Wu, Harp *et al.* 2003).

Although results from these studies suggest that nicotinic receptors can be potential targets of OPs which could lead to selective toxicity, higher concentrations (micromolar) of OPs were generally needed to interact directly with nicotinic receptors, i.e., higher than expected to occur *in vivo(Pope 1999)*.

Muscarinic receptors as targets

Muscarinic receptors have been a focus of neuroscience research because of their role in learning, memory and many other cognitive functions(Levey, Kitt *et al.* 1991; Hersch, Gutekunst *et al.* 1994; Mrzljak, Levey *et al.* 1996; Decossas, Bloch *et al.* 2003; Volpicelli-Daley, Hrabovska *et al.* 2003; Wess 2004; Willets, Nash *et al.* 2004). The search for muscarinic receptor agonists and antagonists is a big area of research and is aimed at discovering and testing drugs directed against specific subtypes of muscarinic receptors so that a desired cholinergic response can be elicited(Roseberry, Bunemann *et al.* 2001; Coulson, Jacoby *et al.* 2002; Liu, Chakraborti *et al.* 2002; Li, Duysen *et al.* 2003; Abdalla, Marostica *et al.* 2004;

Coulson, Jacoby *et al.* 2004; Lee, Fryer *et al.* 2004; Volpicelli and Levey 2004; Wess 2004). Muscarinic agonists and antagonists have been developed and tested in the management of diseases with defects in cholinergic transmission in the brain and in peripheral tissues as well(Yamanishi, Chapple *et al.* 2000; Eglen 2001; Ehlert 2003; Matsui, Griffin *et al.* 2003). For example, disturbances in neurotransmission involving muscarinic receptors have been implicated in the development of Alzheimer's and Parkinson's diseases(Wess 1990; Brown 1996).

Genetically cloned muscarinic receptors are of 5 subtypes namely M1, M2, M3, M4 and M5(Kubo, Fukuda *et al.* 1986; Bonner, Buckley *et al.* 1987; Buckley, Bonner *et al.* 1988). The muscarinic acetylcholine receptors (mAChRs) have 7 transmembrane proteins that are highly conserved among the different subtypes. The muscarinic receptors have 3 loops facing the cytosol and 3 loops facing the extracellular surface. The 3rd intracellular (i3) loop is the most divergent domain(Teber, Kohling *et al.* 2004; Volpicelli and Levey 2004).

Of the 5 muscarinic subtypes, a number of studies have reported that some OPs and their oxons (typically more potent than the parent compound) can bind to M2 muscarinic receptors(Huff, Corcoran *et al.* 1994; Huff and Abou-Donia 1995; Ward and Mundy 1996; Bomser and Casida 2001; Zhang, Liu *et al.* 2002; Fryer, Lein *et al.* 2004), (Huff *et al.* 1994; Betancourt and Carr 2004) at potentially more relevant concentrations.

M2 Acetylcholine Receptors in the Brain

M2 receptors are found throughout the different regions of the brain (Levey *et al.* 1991) and have been localized pre-synaptically in brain areas like the visual cortex(Mrzljak *et al.* 1996), hippocampus (Levey *et al.* 1991) and striatum(Hersch *et al.* 1994). The hippocampal M2

receptor subtype was found to be quite similar to the cardiac M2 receptor subtype(Richards 1990; Ehlert 2003). M2 receptors found in the brain (Gomeza, Shannon *et al.* 1999) and peripheral nerve endings (Bernardini, Roza *et al.* 2002) contribute to antinoception and could be targets in the development of analgesic drugs. M2 knock-out mice did not develop agonist (oxotremorine)-induced tremors and hypothermia(Gomeza *et al.* 1999), implicating this subtype in these pharmacologic/toxicologic responses.

The presynaptic location of M2 receptors in different brain regions suggests an autoreceptor function, regulating the release of ACh from the pre-synaptic terminal(Volpicelli and Levey 2004). Previous studies reported that M2 autoreceptors in rats are already present seven days after birth but do not show full activity until day fourteen(Lee, Nicklaus *et al.* 1990; Won, Liu *et al.* 2001). Both nicotinic and muscarinic autoreceptors exist in the mammalian central nervous system where they modulate the release of acetylcholine. Whereas the function of nicotinic autoreceptors is to stimulate ACh release, muscarinic autoreceptors inhibit ACh release. In superfused brain slices *in vitro*, the active metabolites of parathion and methyl parathion (paraoxon and methyl paraoxon, respectively) decreased Ach release in a concentration-dependent manner, apparently through activation of muscarinic autoreceptors. In contrast, CPO increased release under similar conditions, apparently through blocking these same receptors(Liu *et al.* 2002). *In vivo* CPF exposure inhibited muscarinic autoreceptor function in rat cortical and striatal slices(Won *et al.* 2001). CPF also inhibited the regulatory function of M2 autoreceptors on ACh release in an animal model of airway hypersensitivity. Blockade of M2 autoreceptors in the vagus nerve led to unregulated release of ACh and over-stimulation of post-synaptic M3 receptors in the airway smooth muscles thus causing bronchoconstriction(Fryer *et al.* 2004). Together, these findings indicate that some OPs can

directly and/or indirectly modulate muscarinic autoreceptor function, and this effect could contribute to selective toxicity of anticholinesterases.

M2 Acetylcholine Receptors in Peripheral tissues

In the peripheral tissues, M2 receptors cause muscle contraction in the heart and mediate increased contraction of smooth muscles in the gut, glands and urinary bladder (Yamaguchi, Shishido *et al.* 1996; Goepel, Gronewald *et al.* 1998; Stengel, Gomeza *et al.* 2000; Yamanishi *et al.* 2000) which can lead to excessive glandular secretions and urination(Caulfield and Birdsall 1998; Eglen 2001). M2 receptors are also found in the rat myometrium(Abdalla, Abreu *et al.* 2000; Abdalla *et al.* 2004). The M2 autoreceptors function to regulate the release of acetylcholine from vagus nerves and prevent ACh overstimulation of the M3 receptors in the bronchial smooth muscles (Fryer *et al.* 2004; Lee *et al.* 2004) and ileum(Coulson *et al.* 2002; 2004). Disruption of M2 autoreceptor function could lead to unregulated release and exacerbation of cholinergic toxicity.

Effects of Chlorpyrifos oxon on M2 receptors

Radioligand binding assays have been utilized to measure muscarinic receptor density in different areas of brain and other organs. Chlorpyrifos oxon has been reported to affect binding of muscarinic receptor ligands to receptors in human and rat membranes. In vitro studies reported inhibition of binding of the M2-preferential agonist [^{3}H]cis-methyldioxolane (CD) in rat striatum (IC50 = 22 nM) by chlorpyrifos oxon (CPO) (Huff et al., 1994). CPO also inhibited adenylyl cyclase, the second messenger coupled to M2 receptor at nM concentrations(Huff *et al.* 1994; Huff, Abu-Qare *et al.* 2001). CPO was reported to phosphorylate M2 muscarinic receptors in rat cardiac membranes(Bomser and Casida 2001). Chlorpyrifos oxon, paraoxon and methyl paraoxon, the active metabolites of chlorpyrifos,

parathion and methyl parathion respectively, also decreased M2 receptor binding in membranes from neonatal (IC50 = 15 nM) and adult (IC50 = 7 nM) hearts *in vitro* (Howard and Pope 2002). Exposure of rats to dosages of parathion and chlorpyrifos caused differential changes in muscarinic receptor binding in the brain(Chaudhuri, Chakraborti *et al.* 1993). Exposure to both OPs decreased total muscarinic receptor binding to the nonselective antagonist [^{3}H]quinuclidinyl benzilate (QNB) in the cortex and striatum. In contrast, QNB binding in cerebellum (a region rich in M2 receptors) was decreased by parathion but increased by chlorpyrifos. Similarly, CD binding in all three regions was decreased by parathion but increased by chlorpyrifos. The results of these studies suggest that some OPs could target M2 receptors at concentrations that are physiologically relevant *in vivo,* potentially contributing to selective toxicity. However, the exact mechanism of interaction between OPs and muscarinic receptors is unclear.

M2/M4 receptor regulation

Muscarinic receptors mediate a variety of functions in the brain and peripheral tissues by their interaction with endogenous or exogenous ligands. Interaction of muscarinic receptors with agonists or antagonists can lead to pharmacological or toxicological effects. In order for muscarinic receptors to respond dynamically to rapid changes in agonist concentration, receptor signaling is tightly regulated(Krupnick and Benovic 1998; van Koppen and Kaiser 2003). There are several levels by which muscarinic receptors can be regulated by agonist concentration including changes in receptor density and modulation of transduction pathways However, changes in the receptor itself and not downstream signaling seem to play a more significant role in the regulatory process(Krupnick and Benovic 1998).

Binding assays rely on the relative selectivity of radioligands for receptor subtypes. However, the availability of ligands that specifically bind to the different subtypes is limited and consequently has limited also the elucidation of physiological and pathophysiological events associated with the different subtypes of muscarinic receptors(Wess 2004). Molecular techniques applied to cloning of the genes encoding muscarinic receptors have facilitated a complete characterization of the different subtypes (Kubo *et al.* 1986; Bonner *et al.* 1987; Buckley *et al.* 1988). Polymerase chain reaction (PCR) (Buckley *et al.* 1988; Yamaguchi *et al.* 1996; Rousell, Haddad el *et al.* 1997; Goin and Nathanson 2002; Abdalla *et al.* 2004)has also allowed measurement of mRNA levels of the different subtypes and determination of the rate of receptor synthesis(Yamaguchi *et al.* 1996). Techniques utilizing subtype-specific antibodies and imaging have also allowed mapping of the distribution of the different subtypes of muscarinic receptors in the brain and peripheral tissues(Levey *et al.* 1991; Decossas *et al.* 2003). Knock-out mice lacking a specific muscarinic subtype, AChE and other cellular components (like the kinases) believed to play a vital role in the regulatory pathway have also allowed the characterization of the different physiological processes mediated by muscarinic receptors(Gomeza *et al.* 1999; Walker, Peppel *et al.* 1999; Wess 2000; Duysen, Li *et al.* 2001; Decossas *et al.* 2003; Li *et al.* 2003; Matsui *et al.* 2003; Volpicelli-Daley *et al.* 2003; Wang, Boeck *et al.* 2004; Wess 2004). All of these methods have provided evidence regarding the critical events involved in the regulation of muscarinic receptors.

The binding of an agonist to M2 receptors causes a conformational change in the receptor leading to alteration in cellular activity and gene expression(King 2004). Signaling pathways activated by mAChRs include induction of the activity-regulated cytoskeleton associated gene (ARC) thought to play a role in synaptic plasticity(Teber *et al.* 2004). The

muscarinic M2 and M4 receptor subtypes couple to pertussis toxin-sensitive G proteins, leading to the inhibition of adenylyl cyclase and a consequent decrease in the production of the second messenger cyclic adenosine monophosphate (cAMP)(van Koppen and Kaiser 2003). cAMP activates protein kinase A which in turn phosphorylates other proteins and mediates a variety of cellular responses. Adenylyl cyclase is a coincidence detector and can be activated by signals occurring at the same time. Adenylyl cyclase is believed to be involved in a plethora of physiologica precesses including learning and memory(King 2004).

Desensitization

A rapid decrease in cell signaling in the presence of persistent agonist stimulation was observed for G-protein coupled receptors including muscarinic receptors(Koenig and Edwardson 1997). This event is referred to as desensitization but is also called quenching, deactivation, adaptation and tachyphylaxis(Krupnick and Benovic 1998). Desensitization is characterized by the rapid uncoupling of G proteins from the receptor without a decrease in the total number of receptors.

In HEL 299 cells, long-term treatment with carbachol (12 to 24 hours) caused functional desensitization of M2 receptors measured by agonist-mediated inhibition of cAMP formation(Haddad, Rousell *et al.* 1995). Since M2/M4 receptors are coupled to the inhibition of adenylyl cyclase and accumulation of the second messenger cAMP, increased cAMP levels after agonist treatment can be used as an indicator of receptor desensitization. In control cells, carbachol inhibited forskolin-stimulated cAMP accumulation. However, after 24 hour treatment, the carbachol-induced forskolin-stimulated inhibition of cAMP accumulation was lost (Haddad *et al.* 1995; Rousell, Haddad *et al.* 1995) indicating complete receptor desensitization. Similarly, carbachol lost the ability to inhibit cAMP accumulation in HEL 299

cells pre-treated with pertussis toxin for 4 hours, indicating a critical role for Gi protein. Basal cAMP formation was also increased with chlorpyrifos treatment in neonates, juveniles and adults (Zhang *et al.* 2002) suggesting that in vivo chlorpyrifos led to M2/M4 receptor desensitization. Carbachol and chlorpyrifos oxon have different effects, however on adenylyl cyclase activity in rat striatal tissue, i.e., carbachol inhibited while chlorpyrifos oxon had no effect(Huff *et al.* 2001).

Conversely, the recovery of function of M2 receptors after agonist stimulation was shown to be slow(Haddad, Rousell *et al.* 1995). In HEL 299 cells treated with propylbenzilylcholine mustard (an alkylating agent that binds specifically and irreversibly to cholinergic muscarinic receptors), carbachol regained the ability to inhibit forskolin-stimulated cAMP accumulation only after 12 hours(Haddad *et al.* 1995). However, it took 36 hours for M2 receptor number to return to control levels after removal of propylbenzilylcholine mustard treatment, suggesting slow recovery M2 receptor function(Haddad *et al.* 1995). Similarly, M4 receptors expressed in CHO cells did not regain function for more than 2 hours after removal of carbachol treatment as measured by carbachol-induced forskolin-stimulated cAMP inhibition. Treatment of CHO cells with hypertonic sucrose (450 mM) increased the rate of resensitization(Bogatkewitsch 1996). In cultured neonatal heart cells, carbachol decreased heart cell beating frequency but this effect waned after 1 hour, indicating carbachol-induced desensitization. On the other hand, M2 autoantibodies from patients with idiopathic dilated cardiomyopathy also decreased heart cell beating frequency for more than 5 hours but did not result in desensitization and/or internalization of M2 receptors(Wallukat, Fu *et al.* 1999).

Stimulation by classical and allosteric agonists can affect desensitization(van Koppen and Kaiser 2003). The classical (referred to as the orthosteric) binding site on the M2 receptor

is subject to modulation by the binding of allosteric agonists(van Koppen and Kaiser 2003). The binding of an allosteric modulator brings about a conformational change to the orthosteric site that could enhance or block subsequent binding of classical muscarinic agonists and antagonists. Modulation of agonist binding induced by allosteric modulators, however, is dependent on the muscarinic receptor subtype, the properties of the classical and allosteric ligands and their concentrations(Ellis, Huyler *et al.* 1991; Lee and el-Fakahany 1991; Lazareno and Birdsall 1995; Jakubik, Bacakova *et al.* 1997). Tucek and colleagues reported that the binding of the membrane-impermeable muscarinic antagonist [3H]N-methylscopolamine (NMS) to M2 receptors is affected by alcuronium in a biphasic manner. At low concentrations of alcuronium (10-7 to 10-5 M), NMS binding is increased (positive cooperativity) while at higher concentrations, NMS binding is decreased (negative cooperativity)(Tucek, Musilkova *et al.* 1990). Other compounds like gallamine and strychnine were also reported to alter the binding of muscarinic agonists like carbachol, oxotremorine and pilocarpine(Jakubik *et al.* 1997). Allosteric modulators were also found to possess agonistic action at M2 receptors. Furthermore, allosteric modulators seem not to initiate the same signal transduction pathways as muscarinic agonists (van Koppen and Kaiser 2003). The ability of allosteric modulators to alter subsequent binding of muscarinic agonists could be physiologically important when acetylcholine levels are low or in cases where there is a decrease in the number of cholinergic receptors.

Oxo-M is a radiolabeled agonist reported to bind preferentially to M2 receptors in rat cardiac membranes (Howard and Pope 2002) in vitro. Carbachol (100 pm-5 μM) and the oxons of chlorpyrifos (50 pM-10 μM), methyl parathion and parathion were previously reported to effectively block Oxo-M binding in neonatal and adult rat cardiac

membranes(Howard and Pope 2002). Pre-exposure of the cardiac membranes with CPO prior to extensive washing showed that binding was irreversible(Howard and Pope 2002). In the same light, rats of different age groups treated with low levels of chlorpyrifos showed decreased Oxo-M binding in the cortex(Zhang *et al.* 2002). In another study, treatment of the ileum with acetylcholine (20 μM) showed a decrease in the contractility when subsequently exposed to Oxo-M and prostaglandin F2alpha. This was not observed in M2 receptor knock-out mice. There was also a 3-fold increase in Oxo-M concentration needed to effect contraction.(Griffin, Matsui *et al.* 2004). These studies show that pre-treatment of M2 receptors with an agonist leads to a decrease in the subsequent binding of another agonist (like Oxo-M) with functional impairment of G-protein coupled signal transduction pathways which can be translated as M2 receptor desensitization.

The results of these studies show that CPO can bind to M2 receptors and cause significant changes. However, the exact mechanism by which CPO binds to M2 receptors remains to be fully elucidated. A study showed that radiolabeled CPO diethylphosphorylated M2 receptors in rat cardiac membranes and caused covalent modification of the receptor in vitro(Bomser and Casida 2001). The blockade of Oxo-M binding by CPO pre-treatment with wash-off further supports the idea that CPO caused irreversible covalent modification of the receptor(Howard and Pope 2002). Furthermore, Bomser and colleagues (2001) reported that Oxo-M did not block the binding of radiolabeled CPO suggesting that CPO and Oxo-M bind to different but coupled sites(Bomser and Casida 2001).

Desensitization, the first initial step in muscarinic receptor regulation, is believed to be primarily mediated by G-protein receptor kinases and arrestins (Krupnick and Benovic 1998) and that phosphorylation of M2 receptors is believed to be a key event(DebBurman, Ptasienski

et al. 1995; Pals-Rylaarsdam, Xu *et al.* 1995; Pals-Rylaarsdam and Hosey 1997; Tsuga, Okuno *et al.* 1998). There are two classes of protein kinases that mediate the covalent modification of muscarinic receptors *via* phosphorylation. Second messenger-protein kinases (e.g. PKA and PKC) phosphorylate muscarinic receptors independent of agonist stimulation while serine-threonine G-protein receptor kinases (GRKs) mediate agonist-induced phosphorylation(Hargrave 1994; Hosey 1994; Liggett 1994). Agonist-independent phosphorylation initiates heterologous desensitization whereas agonist-induced phosphorylation initiates homologous desensitization(Hargrave 1994; Hosey 1994; Liggett 1994).

G-protein coupled receptor kinase 2

The G-protein coupled receptor kinases (GRKs) have been shown to facilitate the regulation of G-protein coupled muscarinic receptors. The M2 receptor has been reported as a substrate of G-protein coupled receptor kinase 2 (GRK2; a.k.a. beta2-adrenergic receptor kinase, b2ARK). GRK2 specifically phosphorylates M2 receptors at the i3 loop(Tsuga, Kameyama *et al.* 1994; Pals-Rylaarsdam and Hosey 1997). Although other GRKs have not been shown to phosphorylate M2 receptors, it has been reported that GRK5 knock-out mice are supersensitive to the effects of oxotremorine on M2-mediated cholinergic responses (hypothermia, tremors and salivation) (Gomeza *et al.* 1999)providing evidence that other GRKs may be involved in M2 receptor-mediated functions.

Agonist-specific desensitization is characterized by loss of receptor responsiveness due to uncoupling of G-proteins (DebBurman *et al.* 1995) after agonist stimulation. Agonist-induced receptor phosphorylation, as mediated by GRK2, as the first committed step in receptor desensitization can occur at two phosphorylation sites within the i3 loop, P1 and

P2(Pals-Rylaarsdam and Hosey 1997). Upon agonist stimulation, G-proteins bind to the receptor at the i3 loop where GDP is exchanged for GTP. GTP stimulates Gα to dissociate from Gβγ dimer, the docking protein for GRK2. The binding of the Gβγ subunit is reported to be the limiting step in the phosphorylation of GPCR in cells(Carman, Barak *et al.* 2000).

In the i3 loop of M2 receptors, site P1 is located at residues 286-290 while site P2 is located at residues 307-311 (Figures 3A and 3B), both of which facilitate agonist-induced internalization. However, site P2 is required for the interaction of M2 receptors with beta-arrestin(Pals-Rylaarsdam, Gurevich *et al.* 1997). Deletion of the i3 loop at residues 234-281 prevented M2 receptor internalization (Tsuga, Kameyama *et al.* 1998) (Figure 4A) while deletion of residues at 252-327 prevented agonist-induced desensitization but allowed internalization (Figure 4B). The residues at 282-323 were found to be necessary for M2 receptor phosphorylation but not for internalization(Pals-Rylaarsdam *et al.* 1995).

A. Phosphorylation site P1 (residues at 286-290)

```
241 vkpnnnnmps sddglehnki qngkaprdpv tencvqgeek essndstsvsvasnmrdde
301 itqdentvst slghskdens kqtcirigtk tpksdsctpt nttvevvgss gqngdekqni
361 varkivkmtk qpakkkppps rekkvtrtil aillafiitw apynvmvlin tfcapcipnt
421 vwtigywlcy instinpacy alcnatfkkt fkhllmchyk nigatr
```

B. Phosphorylation site P2 (residues 307-311)

```
241 vkpnnnnmps sddglehnki qngkaprdpv tencvqgeek essndstsvs avasnmrdde
301 itqdentvst slghskdens kqtcirigtk tpksdsctptnttvevvgssgqngdekqni
361 varkivkmtk qpakkkppps rekkvtrtil aillafiitw apynvmvlin tfcapcipnt
421 vwtigywlcy instinpacy alcnatfkkt fkhllmchyk nigatr
```

Figure 3. Phosphorylation sites (P1 and P2).

Phosphorylation sites (P1 and P2) at the i3 loop of the human M2 muscarinic receptor (Pals-Rylaarsdam and Hosey 1997)

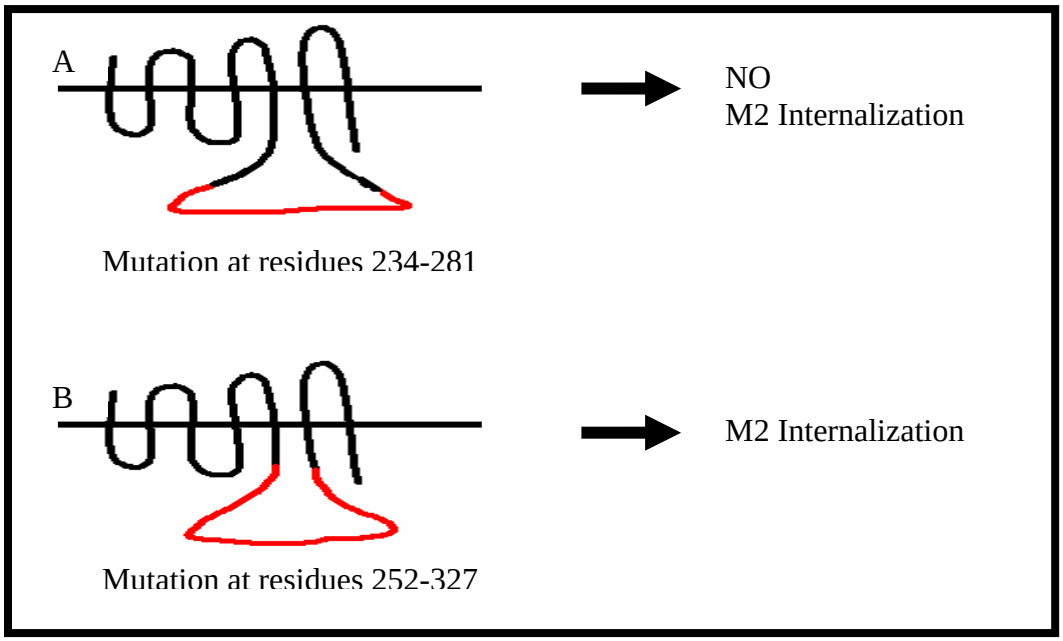


Figure 4. Deletion mutations of residues in the third intracellular (i3) loop.
Deletion by mutation of the residues in the i3 loop led to different effects in M2 receptor internalization. Deletion of residues at 234-281 prevented internalization while deletion of residues at 252-327 allowed internalization but prevented agonist-induced desensitization. Residues at 282-323 were found to be necessary for M2 receptor phosphorylation but not for internalization.
A -(Tsuga *et al.* 1998); B- (Pals-Rylaarsdam *et al.* 1995)

Beta-arrestins

Beta-arrestins are cytosolic proteins and have dual functions in M2 receptor regulation. Beta-arrestins participate in M2 receptor desensitization by binding to the i3 loop of the phosphorylated form of the activated receptor. The beta-arrestin then undergoes dephosphorylation and acts as an adaptor protein for association with clathrin-coated pits(Katz *et al.* 1997). Dynamin associates with clathrin-coated pits, inducing "budding" of pits and the formation of clathrin-coated vesicles (Schlador *et al.* 2000) which leads to M2 receptor internalization.

Beta-arrestin binds to the phosphorylated receptor within regions 307-311 (Lee, Ptasienski *et al.* 2000) of the i3 loop. The i3 loop is reported to be required for internalization

(Tsuga *et al.* 1998) and mutations of residues 307-311 resulted in a loss of receptor/G-protein uncoupling and a loss of arrestin binding (Hosey, Pals-Rylaarsdam *et al.* 1999). The arrestin N-terminal directly participates in arrestin-receptor interaction (Gurevich, Richardson *et al.* 1993) while the C-terminal regulates which type of arrestin binds to the activated and phosphorylated form of the receptor(Gurevich, Pals-Rylaarsdam *et al.* 1997). However, it remains controversial whether beta-arrestins are required for M2 receptor desensitization and internalization or is GRK2 phosphorylation sufficient(van Koppen and Kaiser 2003).

Internalization

Internalization or sequestration was originally defined as the movement of GPCRs from the plasma membrane to subcellular organelles after prolonged agonist-induced stimulation(Krupnick and Benovic 1998). The use of subtype-specific antibodies with imaging techniques as well as membrane-impermeable radioligands like NMS in binding assays have demonstrated the movement of muscarinic receptors from the cell membrane to the intracellular compartment after agonist treatment(Levey *et al.* 1991; Hersch *et al.* 1994; Kurose, Takikawa *et al.* 1998; Roseberry *et al.* 2001; Volpicelli, Lah *et al.* 2001; Delaney, Murph *et al.* 2002; Decossas *et al.* 2003).

As reviewed by Koenig and Edwardson (1997), there are 4 main pathways that determine the intracellular distribution of GPCRs like M2 receptors in the cell: endocytosis, new synthesis, recycling and degradation. Endocytosis is the movement of receptors into the intracellular compartment in a coated vesicle. Newly synthesized receptors are processed by the Golgi complex and targeted to the plasma membrane. After agonist stimulation and endocytosis, receptors are recycled back to the plasma membrane or degraded in the lysosomes(Koenig and Edwardson 1997). Changes in the distribution of receptors after

agonist treatment can be determined by membrane-impermeable radioligands that will bind to surface receptors but cannot pass the cell membrane and bind to receptors in the cytosol (Haddad *et al.* 1995; Bogatkewitsch 1996; Tsuga *et al.* 1998)

Several reports revealed that M2 muscarinic receptor internalization follows agonist-induced GRK2-mediated phosphorylation at the i3 loop. The degree of M2 receptor internalization, which was measured by the loss of the membrane-impermeable radioligand NMS after carbachol treatment in intact cells, was markedly increased (80%) when G-protein receptor kinase 2 (GRK2) was co-expressed in CHO, COS-7 and BHK-21 cells. When the i3 loop was absent in CHO cells and a dominant negative GRK2 was co-expressed in COS-7 and BHK-21 cells, carbachol-induced internalization was blocked in these cells. The half-time (t1/2) of internalization in the presence of 100 μM carbachol in CHO cells was estimated to be 9.5 min(Tsuga *et al.* 1994; Tsuga *et al.* 1998). On the other hand, in CHO cells expressing M4 receptors, receptor internalization was inhibited by pre-treatment with a hypertonic solution of sucrose (450 mM). Return of M4 receptor number to control values occurred 1 hour after carbachol removal as measured by NMS binding(Bogatkewitsch 1996). The time required for internalization of M2 receptors appears markedly dependent on the type of cell studies, however. In HEL 299 cells, carbachol caused loss of NMS binding sites within 12 hours. Long-term carbachol treatment (24 hours) caused 60% loss of NMS binding sites(Haddad *et al.* 1995). In JEG-3 cells transfected with M2 muscarinic receptors, agonist-induced internalization requires amino acids in the carboxyl-terminal end of the i3 loop and the adjacent transmembrane domain(Goldman, Schlador *et al.* 1996). Amino acids flanking the two phosphorylation sites earlier mentioned are implicated in agonist-induced phosphorylation and beta-arrestin-mediated M2 receptor internalization(Lee *et al.* 2000). A study on cultured

neonatal heart cells showed that M2 receptor internalization follows desensitization after carbachol treatment(Wallukat *et al.* 1999). Internalization of M4 receptors expressed in CHO cells was shown to delay resensitization(Bogatkewitsch 1996).

While studies have indicated that agonist-induced GRK2-mediated M2 muscarinic receptor internalization contributes to the loss of response following further agonist stimulation, other studies have shown that desensitization does not lead to internalization in other cell lines(Pals-Rylaarsdam *et al.* 1995; Krupnick and Benovic 1998; Hosey *et al.* 1999). HEK 293 cells expressing dominant-negative allele of GRK2 were not desensitized after carbachol treatment. However, the cells preserved their ability to internalize(Pals-Rylaarsdam *et al.* 1995). Mechanisms independent of GRKs, arrestins, clathrin-coated pits and dynamin have also been shown to facilitate the internalization of M2 receptors in different cell lines(Hosey *et al.* 1999; Vogler, Nolte *et al.* 1999; Schlador, Grubbs *et al.* 2000; Roseberry and Hosey 2001). Furthermore, a study on HeLa cells indicated that M2 receptors could initially internalize via a pathway independent of clathrin but then, at the level of the early endosomes, M2 receptors then followed the clathrin-endocytic pathway(Delaney *et al.* 2002).

Following desensitization and internalization, M2 receptors recycle back to the plasma membrane or are degraded in lysosomes(Koenig and Edwardson 1997). Recycling involves dephosphorylation of the receptor within endosomes by the action of phosphatases(Krupnick and Benovic 1998). Receptor cycling, which is defined as the endocytosis of receptors with continuous agonist stimulation and either migration of receptors back to the cell surface or receptor degradation, is a dynamic event(Koenig and Edwardson 1997). After agonist stimulation with subsequent endocytosis, dephosphorylation reactivates the receptor and through an incompletely characterized pathway, the receptor is transported back to the

extracellular surface or targeted to the lysosomes for degradation(Koenig and Edwardson 1997).

Downregulation as a cumulative effect of OPs

Several studies have shown that downregulation or reduction in density of cholinergic receptors is a compensatory mechanism of tolerance to prolonged AChE inhibition as in the case of OP exposure(Chakraborti, Farrar *et al.* 1993; DebBurman *et al.* 1995; Liu, Olivier *et al.* 1999; Howard and Pope 2002; Zhang *et al.* 2002; Betancourt and Carr 2004; Rhodes, Seidler *et al.* 2004). A number of studies in rat brain have reported the effects of repeated exposure to chlorpyrifos (CPF) and its oxon on muscarinic receptor density. Repeated exposure to low levels of chlorpyrifos caused the same levels of AChE inhibition but caused decreased total muscarinic receptor binding as measured by the membrane-permeable radioligand [3H]quinuclidinyl benzilate (QNB) in adult hippocampus, striatum and cortex(Chakraborti *et al.* 1993; DebBurman *et al.* 1995; Liu *et al.* 1999). Total muscarinic receptor binding in the forebrain was also reduced following repeated exposure to chlorpyrifos in neonates, juveniles and adults(Zhang *et al.* 2002; Betancourt and Carr 2004; Rhodes *et al.* 2004). Muscarinic binding was also decreased in the hippocampus of pups from dams exposed to chlorpyrifos at gestational days 9-12(Qiao, Seidler *et al.* 2004). Rats treated repeatedly with chlorpyrifos from postnatal day 1 to 40 (PND1-40) showed persistent AChE inhibition with a decrease in the total number of muscarinic receptors as measured by QNB binding(Tang, Carr *et al.* 1999).

These studies provide evidence that repeated exposure to some OPs can lead to muscarinic receptor alterations. Although the exact mechanism by which some OPs interact with muscarinic receptors and affect binding is unknown, it has been reported that CPO covalently organophosphorylates M2 receptors in rat cardiac membranes(Huff *et al.* 1994;

Bomser and Casida 2001). The irreversible blockade of the binding of the muscarinic agonist [3H]Oxotremorine-M (Oxo-M) to adult rat cardiac membranes after pre-exposure to chlorpyrifos oxon further supports that some OPs effect covalent binding of muscarinic receptors(Howard and Pope 2002). In AChE knock-out mice, downregulation of M2 and M4 muscarinic receptors was reported to be an adaptive mechanism to the complete absence of AChE(Volpicelli-Daley *et al.* 2003). Interestingly, muscarinic receptor mRNA levels in AChE knock-out mice remain unchanged (Li *et al.* 2003) implying that synthesis of new M2 and M4 muscarinic receptors is unchanged and does not compensate for the lack of AChE in these animals. Therefore, in addition to AChE inhibition which results in prolonged synaptic acetylcholine accumulation leading to an adaptive decrease in muscarinic receptor density, OPs may also covalently phosphorylate muscarinic receptors and thereby contribute to regulation of muscarinic receptors through a direct pathway. Repeated OP exposure could cause direct covalent modification of M2 receptors which is exacerbated by the absence of new M2 receptor synthesis. Covalent modification without receptor synthesis coupled with AChE inhibition due to repeated OP exposure could modulate receptor regulatory pathways and effect long-term, irreversible changes in muscarinic receptor signaling.

Conversely, the management of diseases with deficient cholinergic neurotransmission such as Alzheimer's (AD) and Parkinson's (PD) diseases (Goin and Nathanson 2002; Li *et al.* 2003; Volpicelli-Daley *et al.* 2003; Mac, Correia *et al.* 2004) and other diseases includes the use of anticholinesterases to increase the availability of the neurotransmitter acetylcholine(Knopman 1998; 1998; Wilkinson *et al.* 2004). However, the receptor downregulation in animals lacking AChE (AChE -/-) could potentially influence the efficacy of anticholinesterases as therapeutic agents. To date, the only drugs approved for the

management of AD by the U.S. Food and Drug Authority (US FDA) are anticholinesterases(Van Dyck 2004). Therefore, it is important to study the events in muscarinic receptor regulation in response to anticholinesterases to better understand the physiology of muscarinic receptors and aid in the development of better therapeutic agents.

Hypothesis

Agonist-induced GRK2-mediated phosphorylation of M2 receptor is the first committed step in M2 receptor desensitization(Tsuga *et al.* 1994; Tsuga *et al.* 1998). Therefore, modulation of this crucial step could alter succeeding events in M2 receptor regulation. A study using radiolabeled CPO revealed that CPO diethylphosphorylated M2 receptors in rat cardiac membranes(Bomser and Casida 2001). Previous results in our lab showed that CPO blocked GRK2-mediated phosphorylation of human recombinant M2 receptors *in vitro* (Zou). We therefore hypothesized that **CPO can alter M2 receptor regulation by direct organophosphorylation of the receptor**. Specifically, we postulated that CPO could block GRK2-mediated phosphorylation of M2 receptors, thereby modulating the agonist-induced desensitization and/or internalization. Such direct interaction with the M2 receptor may explain selective differences in toxicity of some OP toxicants in the presence of similar changes in AChE activity.

Objectives

I. Determine the effects of chlorpyrifos oxon (CPO) and the agonist carbachol (CCH), alone and together, on M2 receptor desensitization and internalization using radioligand binding studies in HEL 299 and CHO cells.

II. Evaluate the effects of CPO and CCH, alone and together, on M2 receptor trafficking in striatal primary neurons using immunocytochemistry.

CHAPTER TWO

MATERIALS AND METHODS

Sources of Chemicals

Chlorpyrifos oxon was purchased from Chem Service (West Chester, PA; ≥97% purity). [^{3}H]oxotremorine-M acetate (specific activity 85.8–86.4 Ci/mmol; Oxo-M) and [^{3}H]N-methylscopolamine (NMS specific activity 83.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA). All other chemicals were reagent grade supplied by Sigma Chemical Company (St. Louis, MO). CPO was dissolved in 100% dry ethanol (EtOH) for stock preparation and stored at −70 C until day of the assay. Ethanol concentration in the assays was kept constant at 0.1%. Carbachol was prepared in the media in which the cells were grown.

Cell Culture

Pre-coating with poly-D-lysine

Before cell seeding, 6 or 24-well plates were pre-coated with 1 μg/ml of poly-D-lysine (Sigma, Inc.) in phosphate buffered saline for 2 hours under the hood, rinsed with sterile water and allowed to air-dry under the hood. Likewise, coverslips (13 mm, #1, Fisher, Inc.) were placed inside each well of a 24-well plate and coated with poly-D-lysine using the same method as above.

HEL 299 cells

Cells were grown in Dulbecco Minimum Eagles' Medium (DMEM, Biofluids) supplemented with 10% fetal bovine serum (Hyclone, Inc.), L-glutamine, 100 U/ml of Penicillin, 100 μg/ml of Streptomycin inside a 37 deg C incubator, 5% CO2 and 95% air. The cells were seeded in 6-well plates and grown to confluency before being exposed to drugs.

CHO-K1

CHO-K1 cells stably expressing M2 receptors were graciously provided by Tom Bonner (NIH). The cells were grown in 24-well culture plates (Corning, Inc.) in 0.5 ml of Ham-F12 medium (Invitrogen, Inc.). Ham F-12 medium was prepared by adding 1.6 g NaHCO3 and adding sterile nanopure water to a volume of 500 ml. The pH of the Ham-F12 + NaHCO3 was adjusted to 7.4 before sterile filtration. The F12 medium was supplemented with 10% Fetal Clone II (Hyclone), 10,000 units/ml penicillin and 10 μg/ml streptomycin (Invitrogen, Inc.). Cells that were from passage 1-2 days apart were used since it has been observed that a difference of 3 passages caused a significant change in radioligand binding profile (data not shown) which could confound the specific binding results. Cells that were 90% confluent was used in the study as it was observed that cells that were 60-70% confluent showed significant decrease in receptor binding (data not shown). The cells (passage number 30-31) were seeded in 24-well plates at 200,000 cells per well and grown to confluency (~90%), which usually takes 1-1.5 days, before being exposed to drugs.

Rat Striatal Neurons

Rat striatal neurons were purchased from QBM Cell Science (Vancouver, Canada). Upon arrival, the vials were immediately placed in liquid nitrogen. The protocol for cell culture was based on the company's recommendation with some modifications. On the day of culture, the vial was removed from liquid nitrogen tank and immediately transferred to a 37 deg C water bath for thawing, observing aseptic technique all throughout the process. Vial contents were partially thawed in a 37 deg C water bath for 2-2.5 minutes. After vial contents have been partially thawed, the vial is wiped with tissue moistened with 70% ethyl alcohol and immediately brought inside the laminar flow hood. Aseptic technique was observed all throughout the procedure. The vial contents were transferred to a 50-ml conical tube and 9 ml

of pre-warmed Neurobasal medium (supplemented with B27 without antioxidant, 2 mM L-glutamine, 10 000 units/ml penicillin and 10 μg/ml streptomycin (Invitrogen, Inc.)) was added drop-wise to prevent osmotic shock. The cell suspension was mixed by inverting the 50-ml conical tube twice. An aliquot of the cell suspension was obtained for hemocytometer cell counting. Trypan-blue exclusion dye technique was used to determine cell viability. From the cell suspension, 0.5 ml was seeded in 13 mm poly-D-lysine coated coverslip placed inside each well of the 24-well plate. The cells were incubated at 37 deg C, 5% CO2 and 95% air with an initial medium change after 4 hours. Subsequent medium changes were done 3-4 days. Neurons, at 30 day in culture, were used in the immunocytochemistry experiments.

Experimental Design

Cells were typically exposed to 4 different treatments: vehicle (0.1% ethanol), CPO (100 μM), carbachol (100 μM in most cases) or both CPO and CCH. Two techniques were used to study the potential interactive effects of carbachol and CPO on M2 regulation. First, radioligand binding studies were conducted to determine the proportion of M2 receptors that were desensitized or internalized after treatments. Radioligand binding studies to measure desensitized and internalized receptors were done in human epithelial lung fibroblast (HEL 299, ATCC no. CCL-137), a cell line which naturally expresses M2 receptors. Radioligand binding studies were also conducted using Chinese hamster ovary cells (CHO-K1) stably expressing M2 receptors.

Non-specific binding was measured in the presence of atropine (10 or 100 μM). Specific binding was calculated as the difference between total and non-specific binding and expressed as percent control. Reduction in Oxo-M binding was interpreted as desensitization

while reduction in NMS binding was interpreted as internalization. Data were analyzed using SAS for Randomized Complete Block Design (RCBD) and the Least Significant Difference (LSD) to compare various treatment means. Data were plotted using GraphPad Prism 3.0.

The second technique employed immunocytochemistry to visualize the location of the receptors after treatments in rat striatal cultures. M2 monoclonal antibody (Chemicon, Intl., Temecula, Ca.) raised in rats was used to label the M2 receptors on rat striatal cells. Rhodamine red-x (Jackson Immunoresearch) conjugated to donkey anti-rat was used as the secondary antibody. The receptors were visualized using a Nikon EY600® using a Cy3 filter. Images were captured using a digital camera attached to the fluorescent microscope. Images were taken of cells from 5-10 different random fields and were classified as either punctate or diffuse by someone unaware to the experimental set-up.

Drug Treatments

On the day of the experiment, HEL 299 cells were treated with carbachol (CCH, 100 μM) for 1 hour with or without 15-min pre-exposure to CPO (100 μM) at 37 deg C, 5% CO2 and 95% air. On the other hand, CHO-K1 cells were treated with the same drugs for 1 hour except that for the mix treatment set-up (CPO+CCH), the cells were treated simultaneously with CPO and CCH instead of CPO pre-exposure. Rat striatal neurons were treated with carbachol (CCH, 100 μM) with our without 1 hour pre-exposure to CPO (100 μM). After treatments, cells were washed twice with 0.1 M PBS (pH 7.4). The control set-ups for HEL 299, CHO-M2 and rat striatal cells were treated with vehicle (0.1% ethyl alcohol).

Radioligand Binding Assays

[^{3}H]Oxotremorine (Oxo-M) binding assay in HEL 299 cell membrane preparations

Trypsinization and membrane preparations

After drug treatments, HEL 299 cells were removed from plates by adding 0.5 ml of pre-warmed 0.25% Trypsin-EDTA (Sigma, Inc.) and incubated for 5 minutes at 37 deg C, 5% CO_2 and 95% air. DMEM with serum (0.5 ml) was added to all wells to inhibit the action of trypsin. Trypsinized cells were collected and counted using Neubauer hemocytometer. The total number of cells per ml in the cell suspension was calculated. The cell suspension was transferred to tubes and centrifuged at 200 x g for 4 minutes to remove trypsin and media from the cell suspension. The supernatant was discarded and the cells were resuspended in HEPES assay buffer such that each ml contains 25 x 10^6 cells. To prepare membranes, the cells were homogenized using a Polytron homogenizer on ice at 17, 000 (or 28, 000) rpm for 20 sec twice, with a 30 sec pause in between. The membranes were pelleted by centrifugation at 40, 000 x g for 20 min. The supernatant was discarded and the membrane preparations were resuspended in HEPES buffer.

Aliquots from membrane preparations were transferred to duplicate tubes. Desensitized receptors were measured in membrane preparations using [3H]Oxotremorine-M (Oxo-M). The binding of Oxo-M after treatment with carbachol or CPO with subsequent wash off after treatments is determined by adding 1 nM (Oxo-M, final concentration) to all tubes and allowed to incubate for 1 hour at 21 deg C. Specific binding was calculated as the difference between samples in the presence (non-specific binding) and absence (total binding) of atropine. Loss in specific binding of Oxo-M after pre-treatment with either carbachol or CPO represents the proportion of M2 receptors that lost Oxo-M sensitive binding sites and was interpreted as M2 receptor desensitization. Non-specific binding was measured by adding

atropine 10 μM) to half of the tubes. The reaction was terminated by vacuum filtration with ice-cold buffer over Whatman GF/B (Brandel Inc., Gaithersburg, MD) filter papers saturated with 0.05% polyethylenimine immediately prior to use. Filter disks were soaked overnight in 4.0 ml scintillation cocktail (Scintisafe, FisherBrand, Dallas, TX) and radioactivity measured using Wallac Model 1209 scintillation counter (Perkin–Elmer, Gaithersburg, MD).

[^{3}H]N-methyl scopolamine (NMS) binding assay in intact HEL 299 and CHO-M2 cells

Muscarinic acetylcholine receptor subtype M2 in intact HEL 299 and CHO cells was measured by the membrane-(Bogatkewitsch 1996)impermeable radioligand [^{3}H]NMS. After drug exposure, cells were washed twice with the assay buffer. [^{3}H]NMS, at a final concentration of 2 nM, was added to all the wells and incubated for 1 hour, 37 deg C, 5% CO_2 and 95% air. Non-specific binding was measured by adding atropine (10 μM) to half of the wells. The binding assay was stopped by rapidly aspirating the assay buffer and rinsing the cells with ice-cold PBS three times. Trypsin-EDTA was used to remove HEL 299 cells from the plates while 0.5 ml of 2.5 M NaOH (30 minutes, RT) and 70 ul of 0.25 N HCl (5 minutes, RT) were used to remove CHO-K1 cells from the plates. It is important to swirl the plates after adding NaOH to facilitate cell detachment. Without swirling, it was observed that some CHO-K1 cells remain at the bottom of the plates and affected scintillation counts. The cells were removed from each well and transferred accordingly to 7 ml scintillation vials. Four ml of scintillation fluid, Scintisafe® 30% (Fisher) was added to each vial and radioactivity measured using a Wallac 1209 (Perkin Elmer) scintillation counter. Specific binding was defined as total binding minus non-specific binding and expressed as percent control. Loss in NMS binding after pre-treatment with carbachol or CPO represents the proportion of M2 receptors that were internalized.

Immunocytochemistry

After drug treatments, rat striatal neurons grown on coverslips were rinsed twice with PBS and fixed with freshly-prepared 4% paraformaldehyde-picric acid fixative (PFF) for 20 minutes at room temperature (RT). After fixation, the cells were rinsed three times for 10 minutes each with PBS. The cells were blocked using normal donkey serum (Jackson ImmunoResearch) with 0.2% Triton X-100 and 5% bovine serum albumin (Sigma, Inc.). M2 muscarinic receptors were immunolabeled with the primary antibody rat anti-m2, 1:100 (Chemicon, Intl.) for 18 hours at 4 deg C. After primary immunolabeling, the cells were rinsed three times with PBS, 10 minutes each. The cells were then labeled with donkey anti-rat secondary antibody conjugated to rhodamine red-X (Jackson ImmunoResearch), 1:200 for 1 hour at RT. The coverslips were rinsed with PBS and allowed to air dry at RT. Coverslips were mounted on glass slides (cell side down) using VectaShield as a mounting medium. The cells were viewed using a fluorescent microscope (Nikon Eclipse EY600®). Pictures were taken from 5-10 random fields. A person blinded to the experiment classified the distribution of the receptors as punctate or diffuse. Cells showing punctate or diffuse staining were counted from each treatment and expressed in percent. Punctate staining in the cells represents M2 receptors that are internalized after treatment.

Statistical Analyses

Specific binding was defined as total binding (in the presence of radioligand only) minus non-specific binding (radioligand + atropine) and expressed as percent control and plotted using GraphPad Prism ver 3.0. The data on the effects of vehicle (control), CPO alone (CPO), carbachol alone (CCH) or the mix treatment (CPO+CCH) were analyzed using the SAS ANOVA for a factorial RCBD design. The Least Significant Difference (LSD) to

compare various treatment means was determined. The Chi-Square test was done to compare punctate distribution of M2 receptors from various treatments in the immunocytochemistry experiments.

CHAPTER THREE

RESULTS

Effect of carbachol and CPO on M2 receptor desensitization

To determine the effects of carbachol and CPO on M2 receptor desensitization, HEL 299 cells were exposed to carbachol (100 μM) with or without pre-exposure to CPO (100 μM) with subsequent washing of cells prior to binding analysis. M2 receptors that were desensitized after drug treatments were measured by measuring Oxo-M binding. The proportion of desensitized receptors was expressed as percent control. The rapid loss in Oxo-M binding after carbachol exposure reflects desensitization(Griffin *et al.* 2004). Figure 5 shows that CPO, CCH and the combined exposure caused substantial decrease in Oxo-M binding. Vehicle controls were exposed to 0.1% ethyl alcohol only as described in Materials and Methods. Exposure to 100 μM carbachol for 15 minutes induced a 79% decrease in Oxo-M binding in HEL 299 cells. CPO (100 μM) treatment only caused a 12% reduction. Exposure to both agents led to somewhat greater reduction in binding (85%). Statistical analysis was not done in these data because of the small sample size (n=2) from one experiment.

Effect of carbachol and CPO on M2 receptor internalization

Radioligand binding

To determine the interactive effect of carbachol and CPO on M2 receptor internalization, HEL 299 cells and CHO-K1 cells stably expressing M2 receptors (CHO-M2) were exposed to carbachol with or without CPO. Cells were washed extensively after treatments and surface receptors were measured by NMS binding. In essence, since NMS is a membrane-impermeable muscarinic antagonist, it will only bind to receptors that are on the surface and not to those that are sequestered. Reduction in NMS binding following carbachol

(CCH) exposure relative to vehicle controls reflects agonist-induced receptor internalization. The effects of carbachol and CPO on M2 receptor internalization in the 2 cell lines are shown in Figures 6 and 7.

As shown in Figure 6, carbachol (CCH, 100 μM, 1 hour) reduced NMS binding in HEL 299 cells by 44%. CPO by itself (100 μM, 1 hour) only caused 17% reduction in NMS binding. With exposure to both agents (CPO+CCH), somewhat greater reduction in NMS binding was noted (61%). Again, statistical analysis was not done on these data because of the small sample size (n=2) from one experiment.

In Figure 7, CCH (100 μM) caused a significant loss in NMS binding (39%) in CHO-M2 cells relative to control. CPO alone reduced NMS binding by 16%, but this effect was not statistically significant. With exposure to both agents (CPO+CCH), NMS binding was reduced 19%.

Results in Figures 6 and 7 show that CPO may have different effects on carbachol-induced changes in NMS binding in different cell lines. The effect of a single concentration of CPO (100 μM) on concentration-dependent (10 μM-10 mM) effects of CCH on NMS binding in CHO-M2 cells was also determined. Figure 8 shows the interactive effect of CPO on carbachol-induced loss in NMS binding in CHO-M2 cells and the statistical difference relative to vehicle (control). In the absence of CPO, CCH (10 μM -10 mM) caused a significant loss (35-53%) in NMS binding in CHO cells. CPO appeared to partially block CCH-induced internalization, however. For example, At 100 μM carbachol, inclusion of CPO increased NMS binding from 39% to 19%. At 1 mM carbachol, CPO also decreased carbachol-induced loss in NMS binding from 45% to 25%.

Immunocytochemistry

To evaluate the effects of CCH and CPO on the location of the M2 receptors in rat striatal neurons, M2 receptors were labeled with M2 monoclonal antibody. Fluorescent dye (rhodamine red-x) conjugated to a secondary antibody was used to label the M2 monoclonal antibody. Neurons were viewed under a fluorescent microscope (Nikon EY600) to determine the location of M2 receptors in the cells. Controls included neurons labeled only with the secondary antibody and neurons without any label at all. Treated neurons were viewed under the same settings as neurons in the control set-up.

Figures 9A-D show the fluorescent images taken from rat striatal neurons at 30 day in culture treated with CCH and/or CPO. Immunocytochemistry using rat striatal neurons before 30 days (e.g. at 14 day in culture) did not show evident immunostaining when viewed using confocal microscopy. Cells at 30-day in culture showed immunosignals as shown in the figures and were this condition was therefore used in the experiments. Cells alone did not show autofluorescence (image not shown) nor did the cells labeled only with the secondary antibody (negative control, Figure 9E and 9F). Cells treated with vehicle only showed staining that was diffusely distributed in the cell (Figure 9A). Treatment with CCH (100 μM, 1 hour) revealed strong staining concentrated in punctate dots inside the cells (Figure 9B). Interestingly, CPO-treated cells also showed the same punctate staining that was localized in distinct puncta inside the cells (Figure 9C). Similarly, treatment with both CPO and CCH showed more punctate staining inside the cells (Figure 9D).

Figure 10 shows the percentage of cells showing diffuse or punctate staining after treatment with CPO and CCH. A small percentage of cells (20%) have significant punctate staining under control conditions (vehicle only). An increase in the percentage of cells

showing punctate immunosignals was seen after either CPO (60 %) or CCH (65%) exposure, which were significantly different from controls ($p<0.05$). Treatment with both CPO and CCH showed a greater percentage of cells showing punctate staining (95%) which was also significantly different from controls. Cells treated with CPO or carbachol alone were significantly different in the number of punctate receptors compared to controls, but not different from each other. In contrast, cells treated with both CPO and carbachol showed significantly higher proportion of punctate receptors compared to cells treated with either agent alone ($p<0.05$).

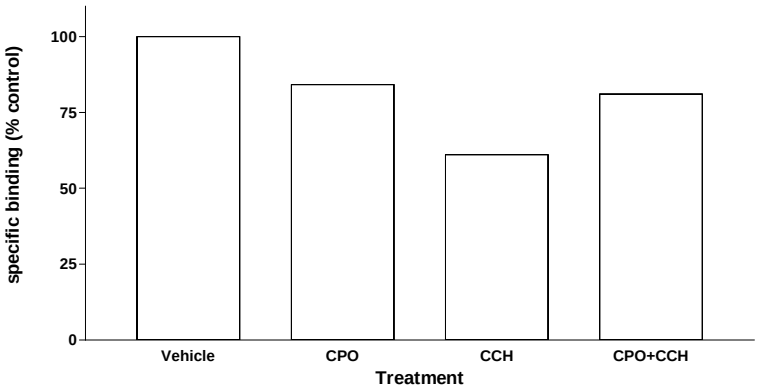


Figure 5. Effect of CPO on [^{3}H]Oxo-M binding in HEL 299 cells

[^{3}H]Oxo-M binding in HEL 299 membranes treated with 100 μM carbachol for 15 minutes with or without 1 hour pre-exposure to 100 μM CPO as described in the Materials and Methods. Each bar represents specific binding expressed as percent of control.

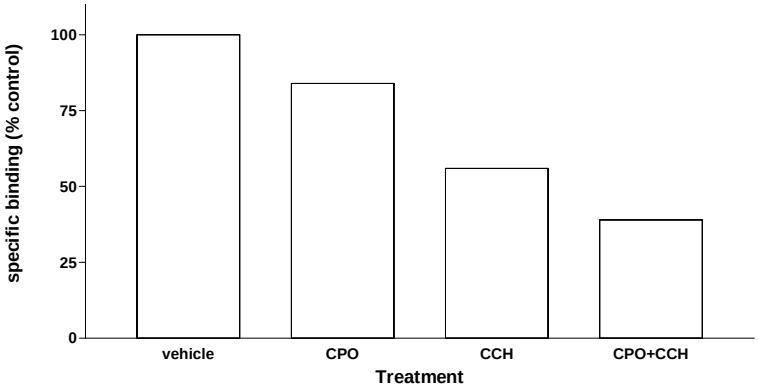


Figure 6. Effect of CPO on [^{3}H]NMS binding in HEL 299 cells

[^{3}H]NMS binding in HEL 299 intact cells treated for 1 hour with 100 μM carbachol with or without 100 μM CPO as described in the Materials and Methods. Each bar represents specific binding expressed as percent of control.

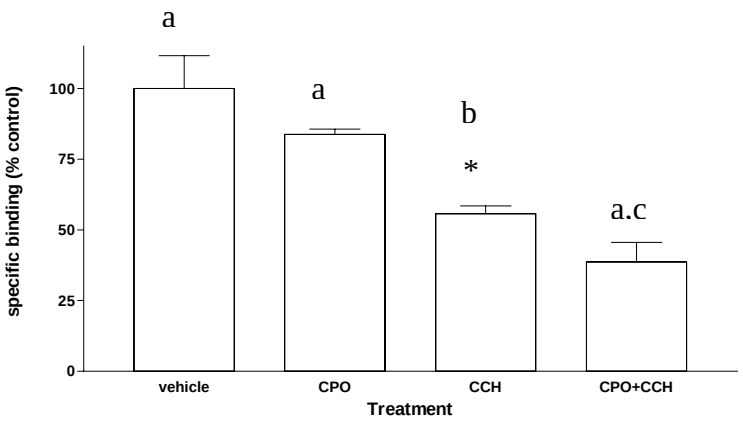


Figure 7. Effect of CPO on [^{3}H]NMS binding in CHO-M2 cells.

[^{3}H]NMS binding in CHO-K1 (CHO-M2) intact cells treated for 1 hour with 100 μM carbachol with or without 100 μM CPO as described in the Materials and Methods. Each bar represents specific binding expressed as percent of control. Asterisks represent values which are statistically significant from vehicle (control) at $p<0.005$. Values with different letters are statistically significant from each other at $p<0.05$ (n=3).

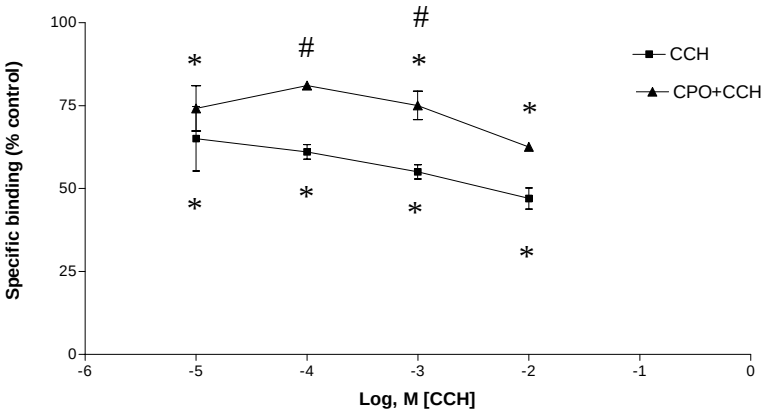


Figure 8. Effect of increasing concentrations of CCH in the presence or absence of CPO on M2 Internalization in CHO-M2 cells.

[^{3}H]NMS binding in CHO-K1 (CHO-M2) intact cells treated with increasing concentrations of CCH (10 μM – 10 mM) with or without 100 μM CPO for 1 hour as described in the Materials and Methods. Each point represents average specific binding from two experiments with n=3 for each experiment. Asterisks represent statistical significance from vehicle (control) at $p<0.05$. Pound sign represents statistical significance on the effect of CPO on carbachol-induced loss in NMS binding ($p<0.05$) (n=3).

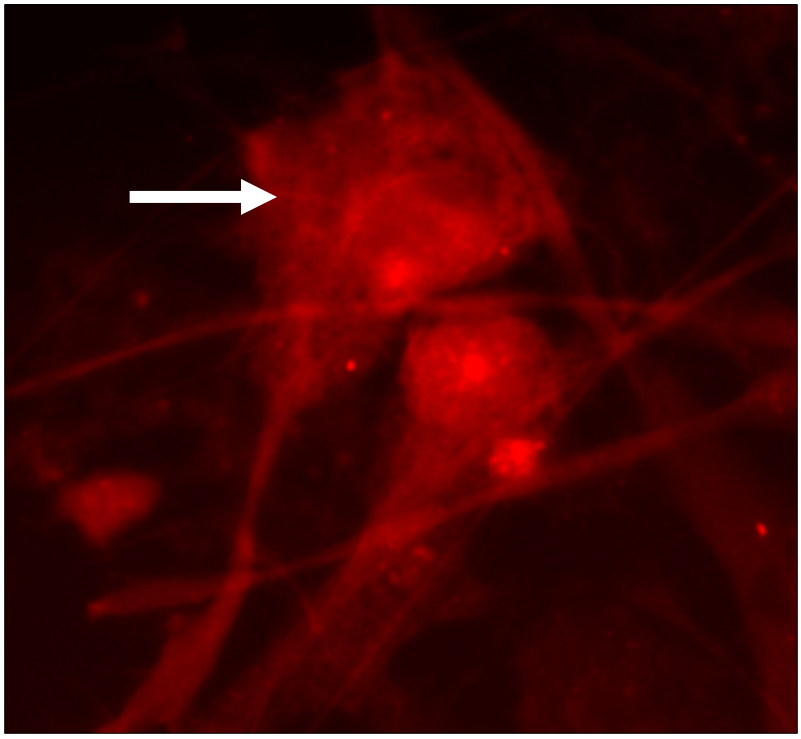


Figure 9A. Effect of Vehicle (control) on M2 receptor internalization in rat striatal cultures (20x).

Rat striatal neurons grown in coverslips were treated with 0.1% Vehicle (control), 100 µM CPO or 100 µM carbachol with or without CPO for 1 hour at 37 deg C, 95% CO_2 and 5% air. Neurons were washed after treatments and fixed with paraformaldehyde-picric acid fixative, blocked and permeabilized with normal donkey serum containing BSA and the detergent Triton-X 100. M2 receptors were labeled with the rat anti-M2 monoclonal antibody for 18 hours at 4 deg C. After primary immunolabeling, cells were washed and then labeled with the donkey anti-rat secondary antibody conjugate to rhodamine red-x for 1 hour. Cells were washed, air-dried and mounted in glass slides using Vectashield as a mounting medium. The cells were viewed under a Nikon fluorescent microscope at 20 x using a light source with a filter for 560 nm excitation wavelength. Cells from random fields were photographed and classified as punctate or diffuse by someone unaware of the treatment conditions. Arrow indicates diffuse staining of M2 receptors in rat striatal cells.

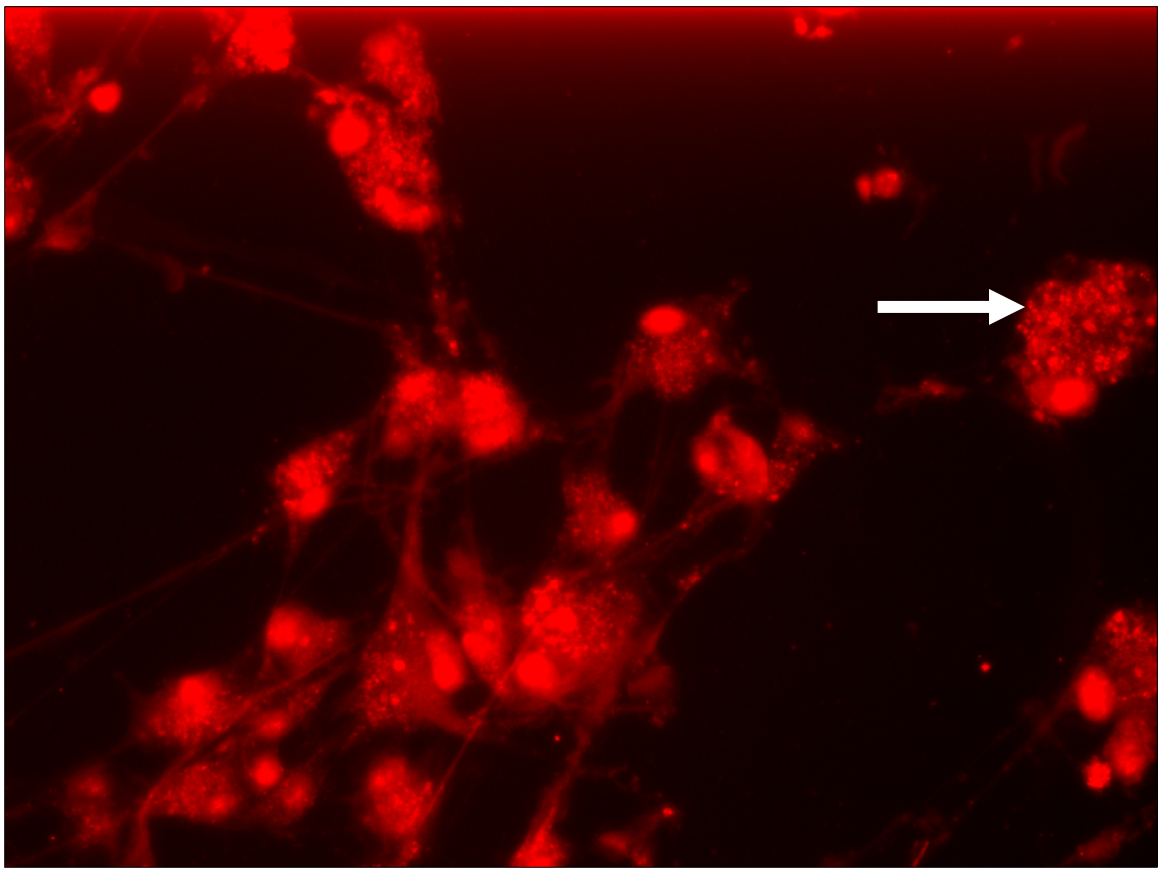


Figure 9B. Effect of CCH on M2 receptor internalization in rat striatal cultures (20x).
Rat striatal neurons treated with carbachol (CCH, 100 μM, 1 hour). Arrow indicates the presence of "punctate" staining.

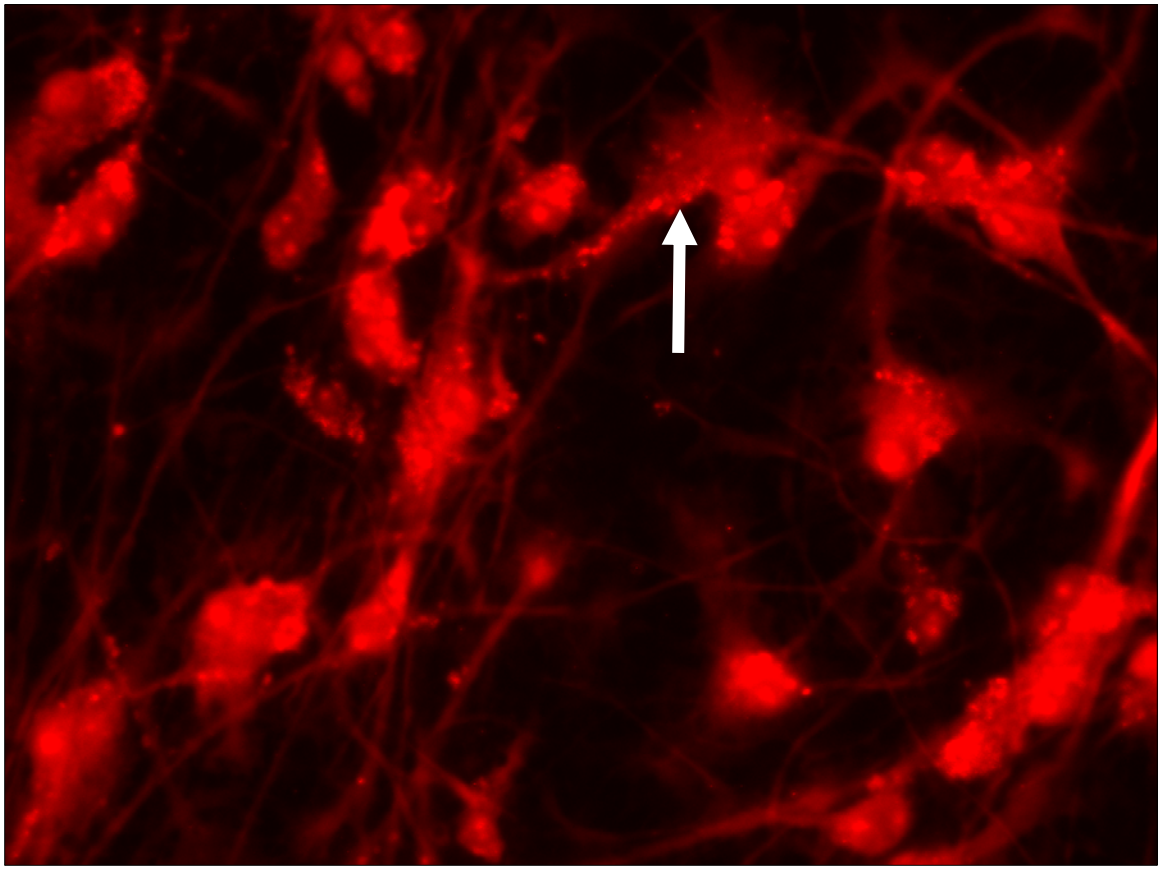


Figure 9C. Effect of CPO on M2 receptor internalization in rat striatal cultures (20x).
Rat striatal neurons treated with CPO (CPO, 100 μM, 2 hours). Arrow indicates the presence of "punctate" staining in the cytoplasm.

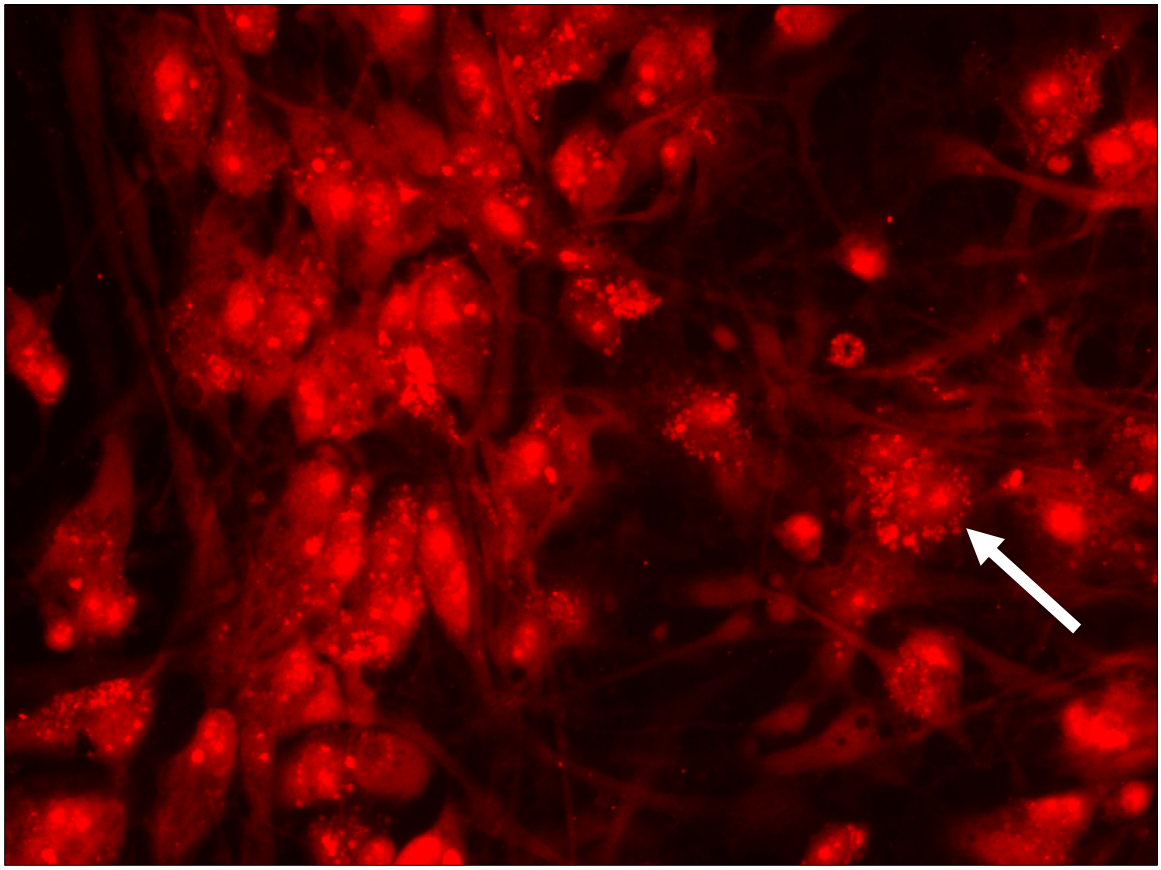


Figure 9D. Effect of CPO+CCH on M2 receptor internalization in rat striatal cultures (20x).

Rat striatal neurons treated with carbachol in the presence of CPO (CPO+CCH). Arrow indicates the presence of "punctate" staining in the cytoplasm.

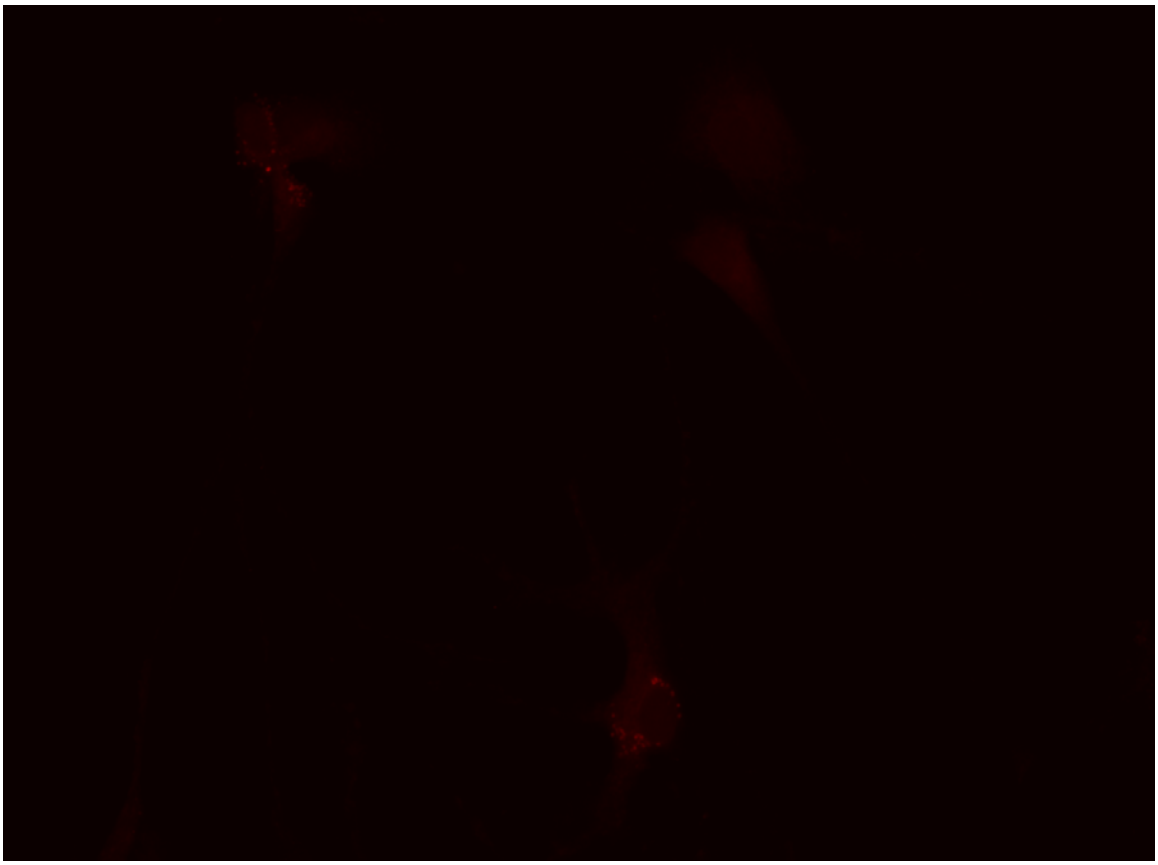


Figure 9E. Negative control (20x)

Untreated rat striatal neurons immunolabeled only with secondary antibody to show background staining.

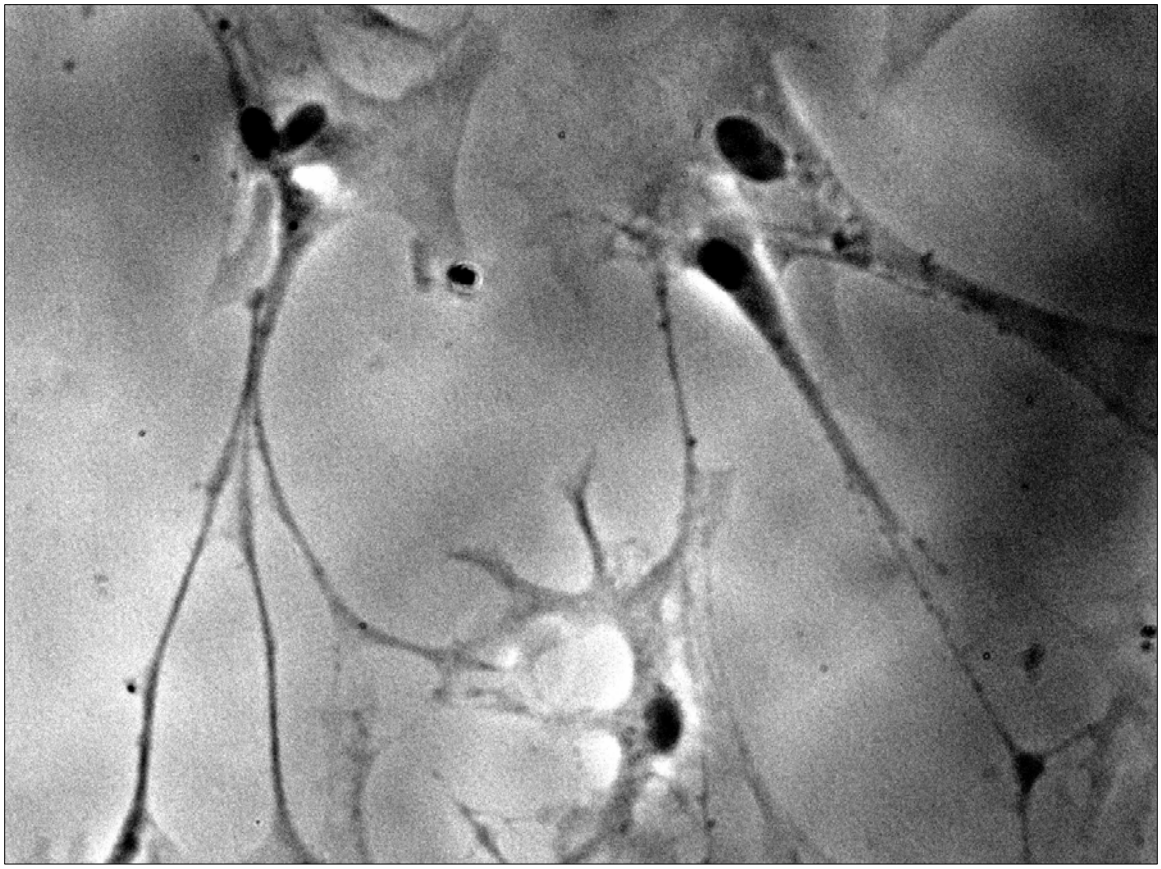


Figure 9F. Negative control (20x)
Untreated rat striatal neurons (bright field)

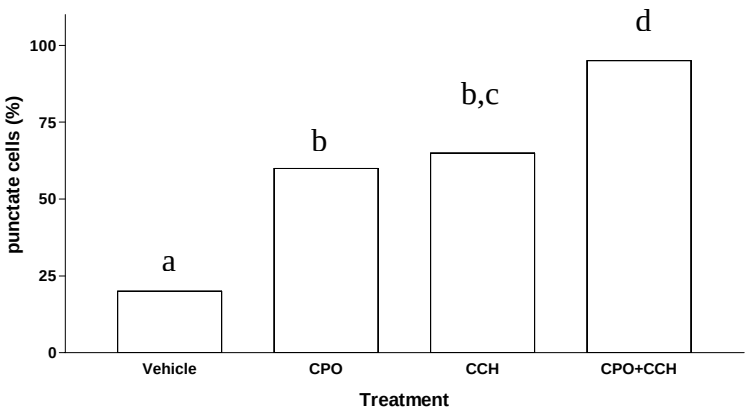


Figure 10. Rat striatal cells showing punctate imunosignals after treatment with vehicle (control) carbachol (CCH) with (CPO+CCH) or without CPO.

Each bar represents percentage of cells counted from each treatment showing punctate immunostaining in 5-10 random microscopic fields. Punctate classification was done by someone unaware of the treatments. Bars with different letters are statistically significant from each other at $p<0.001$.

CHAPTER FOUR

DISCUSSION

Preliminary findings from our laboratory demonstrated inhibition of GRK2-mediated phosphorylation of recombinant M2 receptors by the organophosphate toxicant, chlorpyrifos oxon (CPO). GRK2-mediated phosphorylation of M2 receptors is thought to be the initial step in regulation of these receptors. In the present study, we extended these preliminary observations to evaluate the effects of CPO and the agonist carbachol on two processes in M2 receptor regulation, desensitization and internalization. To investigate whether CPO could alter CCH-induced M2 receptor desensitization and internalization, cells were exposed to carbachol and CPO, alone or together, and radioligand binding studies using the high affinity agonist [^{3}H]oxotremorine-M (Oxo-M) and the membrane impermeable muscarinic antagonist [^{3}H]N-methyl scopolamine (NMS) were conducted. Rapid reduction in binding of the agonist Oxo-M was interpreted as desensitization, whereas a decrease in NMS binding was interpreted as internalization. Immunocytochemistry techniques were also used to visualize the location of M2 receptors in rat striatal neurons after treatment with CCH, CPO or their combination.

In the presence of persistent agonist stimulation, e.g. with prolonged AChE inhibition, cells downregulate their surface receptors in an attempt to maintain homeostasis(Volpicelli-Daley *et al.* 2003). Although several studies have been conducted to elucidate the mechanisms involved in muscarinic receptor downregulation in different cell lines and in knock-out mice(Haddad *et al.* 1995; Hosey, Benovic *et al.* 1995; Pals-Rylaarsdam *et al.* 1995; Barnes, Haddad *et al.* 1997; Koenig and Edwardson 1997; Pals-Rylaarsdam *et al.* 1997; Tsuga *et al.* 1998; Lee *et al.* 2000; Schlador *et al.* 2000; Stengel *et al.* 2000; Werbonat, Kleutges *et al.* 2000; Delaney

et al. 2002; Decossas *et al.* 2003; Li *et al.* 2003; Volpicelli-Daley *et al.* 2003; Wess 2004), the mechanism by which OPs regulate muscarinic receptor regulation is relatively unknown. Thus, while OPs can affect receptor regulation indirectly by elevating endogenous acetylcholine levels, some OPs may also directly target elements of the receptor regulatory pathway potentially leading to selective differences in receptor regulation.

The first committed step in agonist-induced M2 receptor desensitization is the phosphorylation of the activated receptor by GRK2 at the i3 loop. CPO had been reported to covalently modify (diethyphosphorylate) M2 receptors in rat cardiac membranes(Bomser and Casida 2001). However, the phosphorylation site was unknown. The i3 loop of human M2 receptor has been reported to have 2 sites of phosphorylation; namely P1 and P2. P1 is located in the i3 loop at residues 286-290 while P2 is located at residues 307-311. Agonist-induced GRK2-mediated phosphorylation can occur at both phosphorylation sites but site P2 has been reported to be required for M2 receptor-beta-arrestin interaction (Lee *et al.* 2000) leading to clathrin-mediated internalization.

Based on our binding assay results, CCH caused M2 receptor desensitization in HEL 299 cells (Figure 5). Our results showed that the agonist carbachol caused rapid and substantial (79%) loss of Oxo-M binding within 15 min. of exposure. On the other hand, CPO, for a longer time of exposure (1 hour), had minimal effect (12% reduction). We predicted that prior exposure to CPO would block GRK2-mediated phosphorylation of the receptors, such that subsequent exposure to agonist would elicit lesser desensitization. When cells were pre-exposed to CPO prior to CCH treatment to determine if CPO could block carbachol-induced M2 receptor desensitization, however, the findings indicated that CPO did not block carbachol-induced M2 receptor desensitization (Figure 5). In fact, a somewhat greater

reduction in Oxo-M binding (85%) was observed, suggesting possible additive effects between CPO and carbachol on M2 receptor desensitization.

In studies evaluating M2 receptor internalization, our findings using NMS binding as an indicator suggest that CCH indeed leads to internalization of M2 receptors in HEL 299 cells (Figure 6). In CHO-M2 cells, CCH exposure also led to significant reduction in NMS binding. Carbachol-induced M2 receptor internalization was also observed in rat striatal primary neurons using fluorescence microscopy. Intracellular punctate staining was observed in striatal cells treated with carbachol compared to cells treated with vehicle only, where immunosignals appeared diffusely distributed.

When the effect of CPO on carbachol-induced internalization was measured by the loss of membrane-impermeable radioligand NMS binding, our results suggest that CPO, at 100 μM, caused opposite effects on M2 receptor internalization in the two cell lines. In HEL 299 cells, CPO did not block carbachol-induced M2 receptor internalization whereas in CHO-M2 cells, carbachol alone led to significantly greater reduction in NMS binding compared to cells treated with both toxicants. CPO, on its own, did not cause M2 receptor internalization in the two cell lines.

The blockade of carbachol-induced M2 receptor internalization in CHO-M2 cells by CPO was further investigated by exposing CHO-M2 cells to increasing concentrations of CCH in the presence or absence of a single concentration of CPO. While CCH caused a concentration-dependent decrease in NMS binding in the absence of CPO, co-exposure to CPO led to lesser reduction in NMS binding. These results support our original hypothesis that CPO could block initial steps in receptor regulation induced by agonist.

Interestingly, whereas CPO had opposite effects on M2 receptor internalization in the two cell lines using radioligand binding studies, immunocytochemistry results indicated that CPO led to similar prominent staining as punctate regions within the cells as that seen with carbachol treatment alone. Furthermore, while treatment with CPO and carbachol individually increased the proportion of cells exhibiting punctuate staining, combined CPO and carbachol exposure led to a significantly higher proportion of cells exhibiting punctate staining. Our immunocytochemistry results agree with previous studies showing intracellular punctate staining of M2 receptors in rat striatum after treatment with agonists and anticholinesterases(Decossas *et al.* 2003).

Our results generally agree with previous reports on the effect of CCH on NMS binding in CHO cells(Bogatkewitsch 1996; Tsuga *et al.* 1998).One way this could occur is that CCH activation of M2 receptor caused Gβγ to bind to the i3 loop and serve as a docking protein for GRK2 to phosphorylate the activated receptor at site P2 (Figure 11). The uncoupling of the Gα subunit from the Gβγ dimer uncoupled the M2 receptor, preventing further agonist stimulation and leading to M2 receptor desensitization. CCH-induced GRK2-mediated phosphorylation recruited beta-arrestin to site P2 in the i3 loop and served as an adaptor protein for clathrin-mediated internalization. The effect of CCH on M2 internalization was evidenced by loss of NMS binding and the punctate staining of M2 receptors inside the cells as shown by immunocytochemistry studies in rat striatal neurons.

CPO, on the other hand, caused different effects in M2 receptor regulation events in different cell types. CPO caused minimal desensitization in HEL 299 cells compared to carbachol. Based on our results, CPO acted as a partial agonist causing minimal M2 receptor desensitization compared to the full agonistic effect of carbachol on M2 receptor

desensitization. In CHO-M2 cells, CPO by itself did not cause significant M2 receptor internalization as compared to carbachol. However, with the combined treatment (CPO+CCH), CPO apparently blocked carbachol's effect on M2 receptor internalization. In this case, CPO acted like an antagonist at M2 receptors in CHO-M2 cells. It is important to differentiate here between the possibility that CPO actually bound to the ligand binding site on the receptor to act as a partial agonist or antagonist, or whether it bound at a distinct site and elicit regulatory actions mimicking a partial agonist or antagonist. The studies described herein cannot discriminate between these two possibilities.

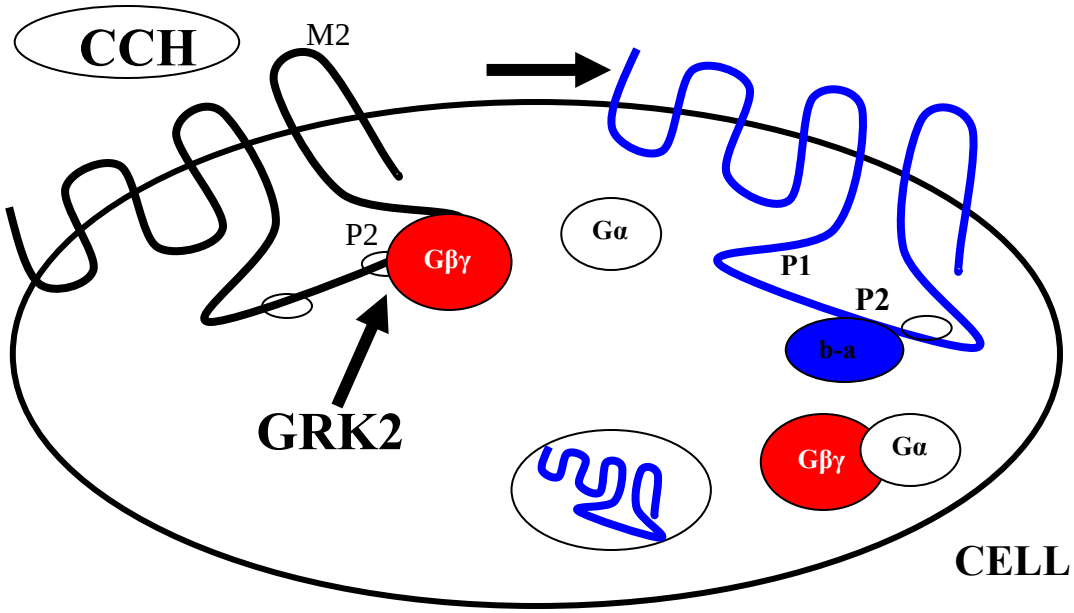


Figure 11. A schematic representation of the events in M2 receptor desensitization and internalization through CCH- induced GRK2-mediated phosphorylation, Gα subunit uncoupling and beta-arrestin binding.

Legend: b-a – beta-arrestin; CCH – carbachol; GRK2 – G-protein coupled receptor kinase 2; Gα – Galpha; Gβγ – G betagamma dimer; M2– M2 muscarinic acetylcholine receptor; P1 – phosphorylation site 1; P2- phosphorylation site 2.

One possible mechanism for differential effects of CPO on M2 desensitization and internalization in different cell lines is the organophosphorylation of site P1 within the i3 loop by CPO, which caused Gα subunit to detach from the M2 receptor in HEL 299 cells (Figure 11). It has been previously reported that CPO diethelphosphorylated rat cardiac membranes(Bomser and Casida 2001). However, the phosphorylation site or the exact mechanism of this event is not fully elucidated. Uncoupling of the Gα subunit caused weak M2 receptor desensitization which prevented further Oxo-M binding. Under these conditions, CPO could have caused M2 receptor desensitization independent of GRK2-mediated phosphorylation.

Furthermore, since site P2 is required for beta-arrestin-mediated endocytosis and not P1, CPO did not cause significant internalization. However, CPO caused apparent M2 receptor internalization in rat striatal cells in the immunocytochemistry studies. One explanation for this is the presence of M2 receptor internalization machinery in primary rat striatal neurons, possibly having more intact regulatory mechanisms compared to cell lines.

When cells were exposed to both CPO and CCH, a greater loss in Oxo-M and NMS binding was observed in HEL 299 cells suggesting apparent additive effects in M2 desensitization and internalization in this cell line. The increase in the percentage of desensitized M2 receptors in the presence of both CCH and CPO further supports the idea that CCH caused GRK2-mediated phosphorylation at site P2 and recruited beta-arrestin leading to internalization while CPO phosphorylated site P1. Other kinases besides GRK2 can potentially play a role in the regulation of M2 receptors in HEL 299 cells as well. Extracellular signal-regulated protein kinases (ERKs), involved in cell growth and differentiation in a number of cell types, can participate in M2 receptor regulation in HEL 299 cells(Rousell *et al.*

1997). Furthermore, CPF and CPO were found to activate ERK 44/42 in CHO cells expressing M2 receptors, with the oxon being 2-3 fold more potent than CPF in activating ERKs(Bomser and Casida 2000). CPO activation or inhibition of other kinases could therefore theoretically alter M2 desensitization in HEL 299 cells and confound the study of CPO-mediated inhibition of M2 phosphorylation by GRK2 and its effects on receptor regulation. Furthermore, the small sample size (n=2) for this single experiment precludes statistical analysis. The effect of CPO on carbachol-induced M2 receptor desensitization in HEL 299 cells is therefore suggestive but inconclusive. However, it has been previously demonstrated that CPO irreversibly blocked the binding of Oxo-M in rat cardiac membranes(Howard and Pope 2002). Replicated experiments with greater sample size would allow clearer interpretation with a higher confidence.

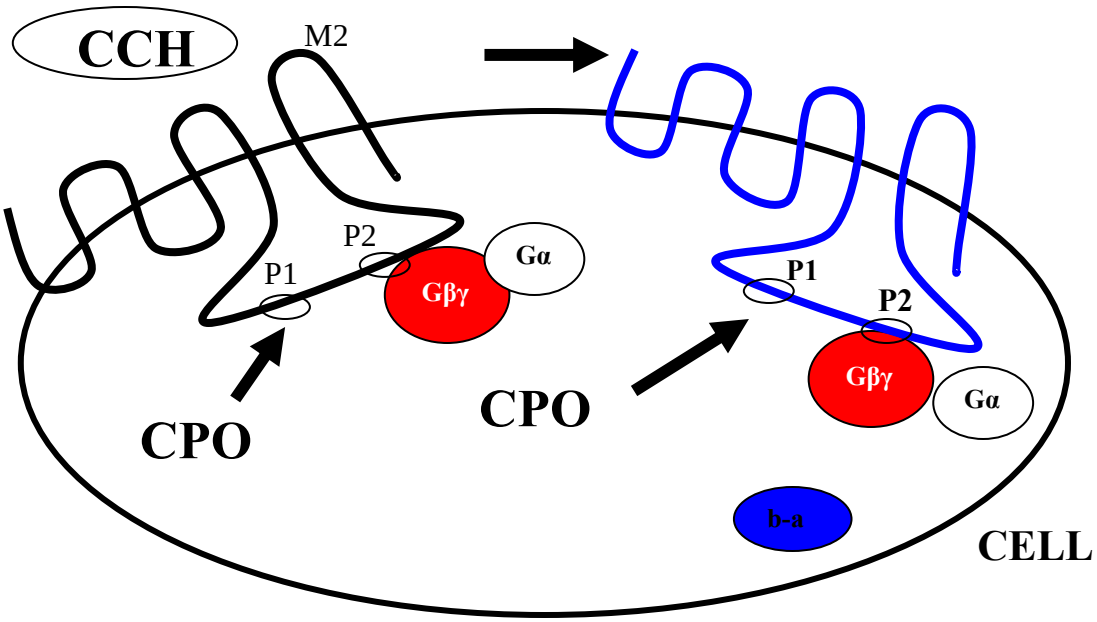


Figure 12. A schematic representation of the events in M2 receptor desensitization through CPO- induced uncoupling of Gα subunit from the M2 receptor.

Legend: CPO-chlorpyrifos oxon; GRK2 – G-protein coupled receptor kinase 2; Gα – Galpha; Gβγ – G betagamma dimer; M2AChR – M2 muscarinic acetylcholine receptor; P1 – phosphorylation site 1; P2- phosphorylation site 2.

In contrast to what was seen in HEL 299 cells, CPO blocked carbachol-induced M2 receptor internalization in CHO-M2 cells. CPO possibly organophosphorylated M2 receptors at site P1 or P2, thereby blocking GRK2-mediated phosphorylation of M2 receptors in CHO-M2 cells. Blockade of the GRK2 phosphorylation site could lead to the blocking of beta-arrestin interaction and inhibition of M2 receptor internalization. Thus, in CHO-M2 cells, CPO impaired agonist-induced internalization of M2 receptors and support our hypothesis that the OP could disrupt receptor regulation through direct interaction with the receptor.

Immunocytochemistry studies of rat striatal neurons showed that CPO, in the presence of CCH, caused an increase in the percentage of cells showing intracellular punctate staining. CPO, in this case, acted like a weak M2 receptor agonist in rat striatal cells. The greater CCH-induced internalization of M2 receptors in the presence of CPO supports the idea that molecules other than beta-arrestin could be involved in CPO-mediated phosphorylation in rat striatal neurons which could be lacking in CHO-M2 cells. Subsequent studies evaluating effects of CPO and carbachol on receptor dynamics using mutant receptors lacking sites P1 and P2 could be informative.

Just recently, it was reported that GRK2 could also mediate phosphorylation-independent receptor regulation of M1 muscarinic receptors through the Gα subunit of G proteins(Willets *et al.* 2004). An alternative mechanism for the inhibition of the GRK2-mediated phosphorylation of human recombinant M2 receptor *in vitro* (Zou and Pope, unpublished) is the binding of CPO to the Gβγ binding site. Blockade of the binding site of Gβγ subunit, which acts as a docking site for GRK2, could prevent GRK2 from phosphorylating the activated receptor and potentially decrease GRK2-mediated M2 receptor phosphorylation as observed previously. In intact cells, even if CPO could bind to the Gβγ

subunit binding site and eliminate the docking site for GRK2, CPO could phosphorylate site P1 and initiate uncoupling of the Gα subunit from M2 receptor leading to receptor desensitization and blockade of Oxo-M from binding to the desensitized receptor.

Interestingly, the M2 monoclonal antibody used in the present studies is directed against the i3 loop of M2 receptor at residues 225-359. However, treatment of striatal neurons with CCH and CPO increased M2 receptor localization in punctate regions within the cells reflecting that the binding site of the M2 monoclonal antibody is distinct from the binding site of CPO and GRK2. Although the 2-dimensional microscope images used in the present work cannot confirm the precise location of the immunosignals within the depth of the neuron, previous studies employing ultrastructural techniques have demonstrated immunolabeled M2 receptors in the rat and monkey striatum (Bernard, Laribi *et al.* 1998; Smiley, Levey *et al.* 1999) co-localizing with subcellular organelles (with perinuclear localization) following exposure to anticholinesterases metrifonate (chronic, MTF), tacrine and physostigmine(Decossas *et al.* 2003). Furthermore, M2 receptors in CHO-K1 cells visualized with immunofluorescence confocal microscopy were shown to also co-localize with endocytic vesicles after carbachol treatment(Tsuga *et al.* 1998).

The present study only showed the effect of CPO on the location of the receptors after treatment employing radioligand binding assays and imaging techniques at a single concentration and a single time point. The use of different concentrations of CPO and different time periods of exposure could further elucidate the effect of CPO on M2 receptor regulation. Furthermore, the use of various biochemical tools including radiolabeled CPO, antibodies to different receptor subunits, fluorescent dye or enzymes, could directly establish the binding site/s of the i3 loop of M2 receptors that may be targeted by CPO. The human

M2 muscarinic acetylcholine receptor has been cloned and protein sequences have been made available online(Bonner *et al.* 1987). M2 autoantibodies in the heart were found not to cause M2 receptor internalization as compared to carbachol (Wallukat *et al.* 1999) making M2 antibodies ideal for blocking phosphorylation and internalization sites. Antibodies directed against protein residues believed to participate in GRK2-mediated M2 regulation could determine if such sites, when blocked by antibody, could block the effects of CPO on carbachol-induced M2 receptor regulation events. Co-localization studies using fluorescent dyes to label cell organelles like endosomes, ER or nucleus would give an idea on the location of M2 receptors inside the cells after treatment.

As noted above, only a single concentration of CPO was used in the present studies to evaluate the possible direct effects of CPO on M2 receptor regulation, and this concentration is exceedingly high compared to that possible with *in vivo* exposure to chlorpyrifos. In preliminary *in vitro* studies evaluating effects of CPO on M2 receptor phosphorylation by GRK2, an IC50 of 70 μM was determined. Our studies thus used a concentration slightly higher than that (100 μM) to probe the possible consequences of inhibition of GRK2 phosphorylation on M2 receptor regulation *in vitro*. We hypothesized that this concentration of CPO could inhibit GRK2-mediated receptor phosphorylation in the intact cells. Although real-life exposures to CPO typically involve much lower concentrations (pM-nM), repeated exposures to lower level OP exposures could potentially lead to accumulative binding of the OP to the receptors. A number of studies have shown that exposure to anticholinesterases such as OPs lead to downregulation of muscarinic receptors in the brain. Thus, one limitation of these studies is the lack of further characterization of the effects of short-term (minutes) or prolonged (days) lower concentrations of CPO on these receptor regulatory mechanisms. It is clear that chlorpyrifos and other OP pesticides modulate muscarinic receptors *in vivo* by

alterations in endogenous acetylcholine and in signal transduction pathways which consequently cause neurochemical and neurobehavioral changes(Chakraborti *et al.* 1993; Huff and Abou-Donia 1995; Liu *et al.* 1999; Betancourt and Carr 2004). However, the relative impact of direct CPO interaction with M2 receptors on desensitization and/or internalization remains unclear.

Implications

Our results show that some OPs can bind to cholinergic receptors and such OP-receptor interactions could participate in M2 receptor regulation. The interaction of chlorpyrifos oxon with M2 receptors in brain and peripheral tissues such as heart could be important in overall expression of OP poisoning and in selective differences in toxicity with different OP toxicants. We have provided evidence that some OPs can potentially alter M2 receptor regulation through direct interaction with the receptor, independent of synaptic acetylcholinesterase inhibition. Knowledge of additional sites of action of OP pesticides in addition to AChE could be important in further understanding interactive toxicity with combined exposures to multiple anticholinesterases and in refining cumulative risk assessments for this class of pesticides. Conversely, knowledge of molecular interactions between OP toxicants and muscarinic receptors could be useful in understanding normal receptor regulatory pathways. As noted, downregulation of muscarinic and other receptors is generally observed with prolonged exposure to agonists. The exact mechanisms and the events involved in M2 receptor regulatory processes are yet to be elucidated. OP toxicants that bind to receptors and modify receptor regulation could be tools in the further evaluation of these regulatory pathways.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

We determined the effects of carbachol and CPO on the early events of M2 muscarinic receptor regulation. We investigated the effects of CPO on CCH-induced desensitization and internalization in cells expressing M2 receptors using radioligand binding assay and immunocytochemistry techniques. Our results revealed that CCH caused rapid desensitization of M2 receptors in HEL 299 cells. CCH also caused M2 internalization in HEL 299 cells and CHO-M2 as measured by radioligand binding assays. Immunocytochemistry studies showed that M2 receptors distributed in distinct puncta inside the cells after carbachol treatment.

CPO caused minimal M2 desensitization as revealed by radioligand binding assays in HEL 299 cells. CPO had minimal effects on receptor internalization in CHO-M2 cells, but led to marked internalization as shown by immunocytochemistry studies in rat striatal neurons. In the presence of the agonist carbachol (CCH), CPO had opposite effects on M2 receptor internalization depending on the cell line. In HEL 299 cells, CPO did not apparently block carbachol-induced M2 receptor internalization while in CHO-M2 cells, CPO blocked CCH-induced internalization. In rat striatal cells, CPO did not block CCH-induced internalization but combined exposure actually led to significantly greater apparent internalization. Thus, the cellular model used markedly influenced the results obtained.

We report here a possible mechanism by which anticholinesterases like CPO can alter M2 receptor regulation and potentially affect the expression of OP toxicity. We hypothesized based on preliminary findings that CPO can block M2 receptor phosphorylation by GRK2

agonist-mediated desensitization or internalization would be impaired following CPO exposure. Results did not support our hypothesis in HEL 299 cells or primary striatal neurons, but did generally support the hypothesis using CHO-M2 cells. We therefore conclude that CPO can bind to the M2 receptor and alter early events in agonist-induced receptor regulation, but further studies are needed to determine the relevance of these OP-receptor interactions and the basis for cell-dependent differences in response.

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CHAPTER SEVEN

APPENDICES

Appendix 1. Raw data

A. The Effect of CCH and CPO on Oxo-M binding (% control) in HEL 299 cells.

Sample	Vehicle	CPO	CCH	CPO+CCH
1	101.37	87.50	21.05	17.14
2	98.63	89.41	21.57	13.36

B. The Effect of CCH and CPO on NMS binding (% control) in HEL 299 cells.

Sample	Vehicle	CPO	CCH	CPO+CCH
1	111.49	81.88	58.51	31.78
2	88.51	85.61	52.90	45.55

C. The Effect of CCH and CPO on NMS binding (% control) in CHO-M2 cells.

Experiment	Sample	Vehicle	CPO	CCH	CPO+CCH
1	1	131.32	57.58	61.53	56.00
	2	99.51	60.75	41.98	97.20
	3	69.17	77.61	73.12	91.40
2	1	63.45	86.81	49.63	72.52
	2	117.56	104.38	60.78	100.92
	3	118.99	117.96	79.50	68.45

D. The Effect of Increasing Concentrations of CCH on NMS binding (% control) in CHO-M2 cells.

Experiment	Sample	V	CPO	CCH-5	CCH-4	CCH-3	CCH-2
1	1	131.3215	57.57859	53.6724	61.52795	63.70765	65.69312
	2	99.51083	60.75103	57.60017	41.9754	35.8895	45.96792
	3	69.16769	77.60593	54.70829	73.11704	59.1756	19.96259
2	1	63.45056	86.81495	74.92235	49.63431	46.84901	37.64152
	2	117.5634	104.3783	73.13896	60.77547	65.264	70.23344
	3	118.9861	117.9641	76.32502	79.50105	59.46298	42.78128

E. The Effect of Increasing Concentrations of CCH in the presence of CPO on NMS binding (% control) in CHO-M2 cells.

Experiment	Sample	V	CPO	CPO+ CCH-5	CPO+ CCH-4	CPO+ CCH-3	CPO+ CCH-2
1	1	131.32	57.58	79.33	56.00	45.82	54.13
	2	99.51	60.75	64.33	97.20	85.79	68.67
	3	69.17	77.61	58.46	91.40	80.80	67.25
2	1	63.45	86.81	74.89	72.52	77.23	59.45
	2	117.56	104.38	60.27	100.92	80.86	79.56
	3	118.99	117.96	107.88	68.45	79.99	46.57

Legend:
CPO = chlorpyrifos oxon
CCH-5 = 10 μM CCH
CCH-4= 100 μM CCH
CCH-3= 1 mM CCH
CCH-2= 10 mM CCH
CPO+CCH-5 = CPO + 10 μM CCH
CPO+CCH-4 = CPO + 100 μM CCH
CPO+CCH-3 = CPO + 1 mM CCH
CPO+CCH-2 = CPO + 10 mM CCH

Appendix 2: Statistical Analyses

A. ANOVA and LSD for NMS binding in CHO-M2 cells

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1

Obs	trt	rep	bind
1	Vehicle	1	100.00
2	CPO	1	65.31
3	CCH-5	1	55.33
4	CCH-4	1	58.87
5	CCH-3	1	52.92
6	CCH-2	1	43.87
7	-5	1	67.38
8	-4	1	81.53
9	-3	1	70.80
10	-2	1	63.35
11	Vehicle	2	100.00
12	CPO	2	103.05
13	CCH-5	2	74.80
14	CCH-4	2	63.30
15	CCH-3	2	57.19
16	CCH-2	2	50.22
17	-5	2	81.01
18	-4	2	80.63
19	-3	2	79.36
20	-2	2	61.86

elmarave
2

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	10	-2 -3 -4 -5 CCH-2 CCH-3 CCH-4 CCH-5 CPO Vehicle
rep	2	1 2

Number of observations 20

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3

The GLM Procedure

Dependent Variable: bind

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4770.033260	477.003326	6.62	0.0045
Error	9	648.072520	72.008058		
Corrected Total	19	5418.105780			

R-Square	Coeff Var	Root MSE	bind Mean
0.880388	12.02988	8.485756	70.53900

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	9	4346.281080	482.920120	6.71	0.0046
rep	1	423.752180	423.752180	5.88	0.0382

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	9	4346.281080	482.920120	6.71	0.0046
rep	1	423.752180	423.752180	5.88	0.0382

The GLM Procedure

t Tests (LSD) for bind

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	9
Error Mean Square	72.00806
Critical Value of t	2.26216
Least Significant Difference	19.196

Means with the same letter are not significantly different.

t Grouping		Mean	N	trt
	A	100.000	2	Vehicle
B	A	84.180	2	CPO

	B		A	C	81.080	2	-4
	B		D	C	75.080	2	-3
	B	E	D	C	74.195	2	-5
F	B	E	D	C	65.065	2	CCH-5
F		E	D	C	62.605	2	-2
F		E	D		61.085	2	CCH-4
F		E			55.055	2	CCH-3
F					47.045	2	CCH-2

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5

The GLM Procedure

t Tests (LSD) for bind

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	9
Error Mean Square	72.00806
Critical Value of t	2.26216
Least Significant Difference	8.5848

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	75.142	10	2
B	65.936	10	1

Legend:
Vehicle
CPO – chlorpyrifos oxon (100 µM)
CCH-5 carbachol (10 µM)
CCH-4 carbachol (100 µM)
CCH-3 carbachol (1 mM)
CCH-2 carbachol (10 mM)
-5 → carbachol (10 µM) + CPO
-4 → carbachol (100 µM) + CPO
-3 → carbachol (1 mM) + CPO
-2 → carbachol (10 mM) + CPO

B. Chi-square test for the distribution of punctate or diffuse staining in rat striatal neurons (%).

B.1. Raw Data

Distribution	Vehicle	CPO	CCH	CPO+CCH
Punctate	20	60	65	95
Diffuse	80	40	35	5

B.2. Pair-wise comparison of the different treatments

Treatments	Chi-square value
Vehicle vs CPO	33.3333333333333 *
Vehicle vs CCH	41.4322250639386 *
Vehicle vs CPO+CCH	115.089514066496 *
CPO vs CCH	NS
CPO vs CPO+CCH	35.1254480286738 *
CCH vs CPO+CCH	28.125 *

* significant at p< or equal to 0.001
NS = not significant

Vehicle vs CCH			
	vehicle	**CCH**	**Total**
Punctate	20	65	85
Diffuse	80	35	115
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 20
        Expected value (E) = (row total x column total) / grand total
                E = (85 x 100) / 200 = 42.5
                Chi-square = (O - E)squared / E
                Chi-square = ((20 - 42.5) **2) / 42.5
                Chi-square = 11.9117647058824
Total chi-square now = 11.9117647058824

Processing row 1, column 2 ...
        Observed value (O) = 65
        Expected value (E) = (row total x column total) / grand total
                E = (85 x 100) / 200 = 42.5
                Chi-square = (O - E)squared / E
                Chi-square = ((65 - 42.5) **2) / 42.5
                Chi-square = 11.9117647058824
```

```
Total chi-square now = 23.8235294117647

Processing row 2, column 1 ...
        Observed value (O) = 80
        Expected value (E) = (row total x column total) / grand total
                E = (115 x 100) / 200 = 57.5
                Chi-square = (O - E)squared / E
                Chi-square = ((80 - 57.5) **2) / 57.5
                Chi-square = 8.80434782608696
Total chi-square now = 32.6278772378517

Processing row 2, column 2 ...
        Observed value (O) = 35
        Expected value (E) = (row total x column total) / grand total
                E = (115 x 100) / 200 = 57.5
                Chi-square = (O - E)squared / E
                Chi-square = ((35 - 57.5) **2) / 57.5
                Chi-square = 8.80434782608696
Total chi-square now = 41.4322250639386

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
Sig. 0.20: chi is greater than or equal to 1.64
Sig. 0.10: chi is greater than or equal to 2.71
Sig. 0.05: chi is greater than or equal to 3.84
Sig. 0.025: chi is greater than or equal to 5.02
Sig. 0.01: chi is greater than or equal to 6.64
Sig. 0.001: chi is greater than or equal to 10.83
```

Degrees of freedom: 1
Chi-square = 41.4322250639386
p is less than or equal to 0.001.
The distribution is significant.

Vehicle vs CPO

	vehicle	**CPO**	**Total**
Punctate	20	60	80
Diffuse	80	40	120
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 20
        Expected value (E) = (row total x column total) / grand total
                E = (80 x 100) / 200 = 40
```

```
                Chi-square = (O - E)squared / E
                Chi-square = ((20 - 40) **2) / 40
                Chi-square = 10
Total chi-square now = 10

Processing row 1, column 2 ...
        Observed value (O) = 60
        Expected value (E) = (row total x column total) / grand total
                E = (80 x 100) / 200 = 40
                Chi-square = (O - E)squared / E
                Chi-square = ((60 - 40) **2) / 40
                Chi-square = 10
Total chi-square now = 20

Processing row 2, column 1 ...
        Observed value (O) = 80
        Expected value (E) = (row total x column total) / grand total
                E = (120 x 100) / 200 = 60
                Chi-square = (O - E)squared / E
                Chi-square = ((80 - 60) **2) / 60
                Chi-square = 6.66666666666667
Total chi-square now = 26.6666666666667

Processing row 2, column 2 ...
        Observed value (O) = 40
        Expected value (E) = (row total x column total) / grand total
                E = (120 x 100) / 200 = 60
                Chi-square = (O - E)squared / E
                Chi-square = ((40 - 60) **2) / 60
                Chi-square = 6.66666666666667
Total chi-square now = 33.3333333333333

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
Sig. 0.20: chi is greater than or equal to 1.64
Sig. 0.10: chi is greater than or equal to 2.71
Sig. 0.05: chi is greater than or equal to 3.84
Sig. 0.025: chi is greater than or equal to 5.02
Sig. 0.01: chi is greater than or equal to 6.64
Sig. 0.001: chi is greater than or equal to 10.83
```

Degrees of freedom: 1
Chi-square = 33.3333333333333
p is less than or equal to 0.001.
The distribution is significant.

Vehicle vs CPO+CCH			
	vehicle	**CPO+CCH**	**Total**
Punctate	20	95	115
Diffuse	80	5	85
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 20
        Expected value (E) = (row total x column total) / grand total
                E = (115 x 100) / 200 = 57.5
                Chi-square = (O - E)squared / E
                Chi-square = ((20 - 57.5) **2) / 57.5
                Chi-square = 24.4565217391304
Total chi-square now = 24.4565217391304

Processing row 1, column 2 ...
        Observed value (O) = 95
        Expected value (E) = (row total x column total) / grand total
                E = (115 x 100) / 200 = 57.5          Chi-square = (O -
E)squared / E
                Chi-square = ((95 - 57.5) **2) / 57.5
                Chi-square = 24.4565217391304
Total chi-square now = 48.9130434782609

Processing row 2, column 1 ...
        Observed value (O) = 80
        Expected value (E) = (row total x column total) / grand total
                E = (85 x 100) / 200 = 42.5
                Chi-square = (O - E)squared / E
                Chi-square = ((80 - 42.5) **2) / 42.5
                Chi-square = 33.0882352941176
Total chi-square now = 82.0012787723785

Processing row 2, column 2 ...
        Observed value (O) = 5
        Expected value (E) = (row total x column total) / grand total
                E = (85 x 100) / 200 = 42.5
                Chi-square = (O - E)squared / E
                Chi-square = ((5 - 42.5) **2) / 42.5
                Chi-square = 33.0882352941176
Total chi-square now = 115.089514066496

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
Sig. 0.20: chi is greater than or equal to 1.64
```

```
Sig. 0.10: chi is greater than or equal to 2.71
Sig. 0.05: chi is greater than or equal to 3.84
Sig. 0.025: chi is greater than or equal to 5.02
Sig. 0.01: chi is greater than or equal to 6.64
Sig. 0.001: chi is greater than or equal to 10.83
```

Degrees of freedom: 1
Chi-square = 115.089514066496
p is less than or equal to 0.001.
The distribution is significant.

CPO vs CCH

	CPO	CCH	Total
Punctate	60	65	125
Diffuse	40	35	75
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 60
        Expected value (E) = (row total x column total) / grand total
                E = (125 x 100) / 200 = 62.5
                Chi-square = (O - E)squared / E
                Chi-square = ((60 - 62.5) **2) / 62.5
                Chi-square = 0.1
Total chi-square now = 0.1

Processing row 1, column 2 ...
        Observed value (O) = 65
        Expected value (E) = (row total x column total) / grand total
                E = (125 x 100) / 200 = 62.5
                Chi-square = (O - E)squared / E
                Chi-square = ((65 - 62.5) **2) / 62.5
                Chi-square = 0.1
Total chi-square now = 0.2

Processing row 2, column 1 ...
        Observed value (O) = 40
        Expected value (E) = (row total x column total) / grand total
                E = (75 x 100) / 200 = 37.5
                Chi-square = (O - E)squared / E
                Chi-square = ((40 - 37.5) **2) / 37.5
                Chi-square = 0.166666666666667
Total chi-square now = 0.366666666666667

Processing row 2, column 2 ...
        Observed value (O) = 35
```

```
        Expected value (E) = (row total x column total) / grand total
                E = (75 x 100) / 200 = 37.5
                Chi-square = (O - E)squared / E
                Chi-square = ((35 - 37.5) **2) / 37.5
                Chi-square = 0.166666666666667
Total chi-square now = 0.533333333333333

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
```

Degrees of freedom: 1
Chi-square = 0.533333333333333
For significance at the .05 level, chi-square should be greater than or equal to 3.84.
The distribution is not significant.
p is less than or equal to 1.

CPO vs CPO+CCH

	CPO	CPO+CCH	Total
Punctate	60	95	155
Diffuse	40	5	45
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 60
        Expected value (E) = (row total x column total) / grand total
                E = (155 x 100) / 200 = 77.5
                Chi-square = (O - E)squared / E
                Chi-square = ((60 - 77.5) **2) / 77.5
                Chi-square = 3.95161290322581
Total chi-square now = 3.95161290322581

Processing row 1, column 2 ...
        Observed value (O) = 95
        Expected value (E) = (row total x column total) / grand total
                E = (155 x 100) / 200 = 77.5
                Chi-square = (O - E)squared / E
                Chi-square = ((95 - 77.5) **2) / 77.5
                Chi-square = 3.9516129032258>Total chi-square now =
7.90322580645161

Processing row 2, column 1 ...
        Observed value (O) = 40
        Expected value (E) = (row total x column total) / grand total
                E = (45 x 100) / 200 = 22.5
```

```
                Chi-square = (O - E)squared / E
                Chi-square = ((40 - 22.5) **2) / 22.5
                Chi-square = 13.6111111111111
Total chi-square now = 21.5143369175627

Processing row 2, column 2 ...
        Observed value (O) = 5
        Expected value (E) = (row total x column total) / grand total
                E = (45 x 100) / 200 = 22.5
                Chi-square = (O - E)squared / E
                Chi-square = ((5 - 22.5) **2) / 22.5
                Chi-square = 13.6111111111111
Total chi-square now = 35.1254480286738

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
Sig. 0.20: chi is greater than or equal to 1.64
Sig. 0.10: chi is greater than or equal to 2.71
Sig. 0.05: chi is greater than or equal to 3.84
Sig. 0.025: chi is greater than or equal to 5.02
Sig. 0.01: chi is greater than or equal to 6.64
Sig. 0.001: chi is greater than or equal to 10.83
```

Degrees of freedom: 1
Chi-square = 35.1254480286738
p is less than or equal to 0.001.
The distribution is significant.

CCH vs CPO+CCH

	CCH	CPO+CCH	Total
Punctate	65	95	160
Diffuse	35	5	40
Total	100	100	200

```
Degrees of freedom (df) = (rows - 1) x (columns - 1)
        df = (2-1) x (2-1) = 1

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
        Observed value (O) = 65
        Expected value (E) = (row total x column total) / grand total
                E = (160 x 100) / 200 = 80
                Chi-square = (O - E)squared / E
                Chi-square = ((65 - 80) **2) / 80
                Chi-square = 2.8125
Total chi-square now = 2.8125

Processing row 1, column 2 ...
```

```
        Observed value (O) = 95
        Expected value (E) = (row total x column total) / grand total
                E = (160 x 100) / 200 = 80
                Chi-square = (O - E)squared / E
                Chi-square = ((95 - 80) **2) / 80
                Chi-square = 2.8125
Total chi-square now = 5.625

Processing row 2, column 1 ...
        Observed value (O) = 35
        Expected value (E) = (row total x column total) / grand total
                E = (40 x 100) / 200 = 20
                Chi-square = (O - E)squared / E
                Chi-square = ((35 - 20) **2) / 20
                Chi-square = 11.25
Total chi-square now = 16.875

Processing row 2, column 2 ...
        Observed value (O) = 5
        Expected value (E) = (row total x column total) / grand total
                E = (40 x 100) / 200 = 20
                Chi-square = (O - E)squared / E
                Chi-square = ((5 - 20) **2) / 20
                Chi-square = 11.25
Total chi-square now = 28.125

Calculating probability (P) ...
Looking up critical values for chi at df = 1:
        Sig levels:    0.20 0.10 0.05 0.025 0.01 0.001
        Crit vals:     1.64 2.71 3.84 5.02 6.64 10.83
Sig. 0.20: chi is greater than or equal to 1.64
Sig. 0.10: chi is greater than or equal to 2.71
Sig. 0.05: chi is greater than or equal to 3.84
Sig. 0.025: chi is greater than or equal to 5.02
Sig. 0.01: chi is greater than or equal to 6.64
Sig. 0.001: chi is greater than or equal to 10.83
```

Degrees of freedom: 1
Chi-square = 28.125
p is less than or equal to 0.001.
The distribution is significant.

VITA

Elmar Mabunga Udarbe

Candidate for the Degree of

Master of Science in Veterinary and Biomedical Sciences

Thesis: "The Effect of Chlorpyrifos Oxon on M2 Muscarinic Acetylcholine Receptor Trafficking"

Major Field: Veterinary and Biomedical Sciences

Biographical: Born on November 1, 1976 in Davao City, Philippines, the eldest daughter of Marvelino Bucala and Elsie Mabunga Udarbe among three children (Engineer Leif Marvin and Marielle) and married to Dr. Rex Rhoderick Cerezo Zamora of Petaluma, California, USA, the son of Dr. Emilio Mislang and Amparo Cerezo Zamora and brother to Ms. Zandra Maria Cerezo Zamora.

Education: Graduated from Davao City High School, Davao City Philippines as class valedictorian in April 1993; received Doctor of Veterinary Medicine (DVM) degree from the College of Veterinary Medicine (CVM), University of the Philippines at Los Baños (UPLB) in April 1999 and ranked 5th in her class; completed the requirements for the Master of Science in Veterinary and Biomedical Sciences in July 2004.

Experience: Worked as a student assistant at UPLB; served as an administrative assistant at a private review center for Veterinary Medical Licensure Examination (VMLE); currently employed as an assistant professor at the University of the Philippines-Mindanao; worked as a veterinarian in a private mixed-animal practice in the Philippines and was involved in comprehensive planning for livestock development in Mindanao, Philippines.

Professional Memberships: Society of Toxicology (SOT), International Neurotoxicology Association (INA), Philippine Veterinary Medical Association (PVMA), Philippine Society of Animal Scientists (PSAS), Philippine Society for Microbiology (PSM).

Name: Elmar M. Udarbe, D.V.M. Date of Degree: July, 2004

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECT OF CHLORPYRIFOS OXON ON M2 MUSCARINIC ACETYLCHOLINE RECEPTOR REGULATION

Pages in Study: 90 Candidate for the Degree of Master of Science

Major field: Veterinary Biomedical Sciences

Scope and Method of Study: A primary mechanism of tolerance to anticholinesterases is downregulation of muscarinic receptors. Activation of muscarinic receptors leads to receptor regulation *via* a sequential pathway involving desensitization, internalization and downregulation. The initial step in M2 receptor desensitization is phosphorylation by G-protein Receptor Kinase 2 (GRK2). Our laboratory previously noted that the potent anticholinesterase chlorpyrifos oxon (CPO) can inhibit agonist-mediated phosphorylation of human recombinant M2 receptors by GRK2 *in vitro* while paraoxon, another structurally-related anticholinesterase, had no effect. We hypothesized that CPO can disrupt M2 receptor regulation through direct binding to the M2 receptor at the GRK2-mediated phosphorylation site. To test the hypothesis, the binding of a radiolabeled muscarinic agonist and surface-selective antagonist were measured in cell lines expressing M2 receptors. Immunocytochemistry using M2 subtype-specific antibody was also done to determine the location of M2 receptors after treatments with carbachol and CPO in rat striatal neurons.

Findings and Conclusions: In HEL 299 cells (fibroblasts expressing exclusively M2 receptors), the muscarinic agonist carbachol (CCH) reduced specific binding to the high affinity agonist [^{3}H]oxotremorine-M (Oxo-M, 79%, 15 min exposure) and the extracellular surface-selective antagonist [^{3}H]N-methyl scopolamine (NMS, 44%, 1 hour exposure), suggesting rapid agonist-induced desensitization and internalization. CPO (100 μM, 1 hour) had minimal effect on Oxo-M or NMS binding in the absence or presence of CCH. In CHO cells stably expressing M2 receptors (CHO-M2), CCH decreased NMS binding in a concentration dependent manner. CPO (100 μM) had little effect on its own but impaired carbachol-induced internalization in CHO-M2 cells. In primary neurons, M2 immunosignal was distributed diffusely throughout the cell. CCH (100 μM) led to internalization and concentration of receptors near the cell body. CPO (100 μM) had similar effects as CCH and combined exposure to both led to more extensive internalization. Our results suggest that although CPO may inhibit GRK2-mediated M2 phosphorylation, CPO differentially affects receptor regulation in different cells expressing M2 receptors. Direct binding of CPO to muscarinic M2 receptors could, however potentially affect receptor regulation pathways *in vivo* and contribute to cholinergic toxicity and tolerance to anticholinesterases.

Advisor's Approval: DR. CAREY N. POPE_____