CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

Department of Biochemical Sciences

Interactions of muscarinic receptors and choline esterases:

Functional examinations of esterase inhibitors in the rat.

Dissertation Thesis

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Declaration

I declare that this dissertation thesis is my own, unaided work. All literature and other sources that I used in the thesis are named in References and cited properly. The thesis was not used for obtaining a different or same degree.

Prohlášení

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Hradec Králové Uday Kumar Killi

Dedicated to My Professors, My Family and

Teachers, My Friends, Well wishers

Abstract

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Title of Doctoral Thesis: **Interactions of muscarinic receptors and choline esterases: Functional examinations of esterase inhibitors in the rat.**

The parasympathetic nervous system regulates a number of vital body functions. The neurotransmission within the parasympathetic nervous system is exerted by acetylcholine, a phylogenetic old neurotransmitter, which acts on nicotinic and muscarinic receptors. Whilst the nicotinic receptors are located in the ganglia, the muscarinic receptors are located on the glands and smooth muscles. The muscarinic receptors belong to the large group of G glycoprotein-coupled receptors. Five subtypes exist of the muscarinic receptor (M1-M5), which can be either excitatory or inhibitory depending on subtype. Within the synaptic cleft, acetylcholinesterase hydrolyses acetylcholine and the inhibition of acetylcholinesterase, by for instance pesticides, causes accumulation of acetylcholine at the synaptic cleft, which in turn causes overstimulation of cholinoceptors.

Muscarinic receptors and the acetylcholinesterase are important therapeutic targets in many disorders such as neurodegenerative disorders, disorders of the lower urinary tract, of the cardiovascular and of the respiratory tract. Muscarinic antagonists are sometimes used in drug therapies. However, because of the highly conserved orthosteric (primary binding) domain of muscarinic receptors they lack receptor subtype selectivity, which would be a beneficial feature in the perspective of adverse effects. Allosteric (secondary binding sites) modulators, which act on muscarinic receptor subtypes, have been developed and they may provide new possibilities

in future drug development. One drug of interest is obidoxime, which is suggested to be an allosteric modulator and which combines both esterase inhibition and antimuscarinic effects.

In the present thesis, three types of compounds have been used – a potent cholinesterase inhibitor (physostigmine), weak cholinesterase inhibitors which express muscarinic receptor antagonism (e.g. obidoxime) and a "selective" muscarinic M2 receptor antagonist (methoctramine). Obidoxime has a M2 "selective" antagonistic profile. The goal of the thesis was to determine the functional significance of muscarinic M2/M3 receptors in the state of acetylcholinesterase inhibition. The frequent occurrence of muscarinic M2 receptors on the smooth muscle within the respiratory and lower urinary tract has puzzled researchers for many years. Now, the pharmacological tools employed in the current thesis enabled us to study and discover new interaction mechanisms between muscarinic M2 and M3 receptors, which may be of utter significance for toxicity problems in the pharmacotherapy of esterase inhibition.

Methods: The functional studies were performed on isolated organs (rat atrium and urinary bladder strips) using *in vitro* organ bath experiments. In *in vivo* experiments, heart rate and urinary bladder pressure were studied in anaesthetized rats.

Key Findings: The results confirms that obidoxime exerts anti-muscarinic effects and that the muscarinic receptor inhibition profile shows M2 receptor selectivity. This antimuscarinic effect is much smaller than the effect of atropine. The results also indicate that the esterase inhibition and the muscarinic receptor antagonism occur at different concentrations and dose levels.

The current results also suggest a new role for the muscarinic M2 receptors, namely that they, at least at low intensity of cholinergic stimulation, also stabilize the bladder in the way of not being too sensitive towards acetylcholine. The findings should be considered when administering compounds that enhance cholinergic effects simultaneously with exerting muscarinic M2 receptor antagonism. Consequently, when using acetylcholinesterase reactivators, it seems reasonable to administer an oxime reactivator with less pronounced muscarinic M2 receptor affinity.

Conclusions: The present thesis shows that stimulation of muscarinic M2 receptors inhibits muscarinic M3 receptor-evoked contractile responses at low concentrations of acetylcholine in the synaptic cleft. The toxic effect of cholinesterase reactivators may be coupled to the previously described antimuscarinic M2 receptor profile. The muscarinic M2 and M3 receptor

crosstalk could thus be a counteracting mechanism in the treatment of acetylcholinesterase inhibition when using reactivators, such as obidoxime.

Key words: Atria, Urinary Bladder, Cholinoceptors, Acetylcholinesterase, Muscarinic Receptors, Acetylcholinesterase Inhibitors, Physostigmine, Obidoxime, Muscarinic Antagonists, Methoctramine, Atropine, Crosstalk, Muscarinic Agonists, Methacholine, Acetylcholine.

Abstrakt

Univerzita Karlova v Praze

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Katedra biochemických věd

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Název disertační práce: **Interakce muskarinových receptorů a cholinesterasy: Studie funkcí inhibitorů esterasy u potkana.**

Parasympatický nervový systém reguluje životně důležité tělesné funkce přes cholinergní přenos. Acetylcholin, fylogeneticky starý neurotransmiter, působí na nikotinové receptory v gangliích a muskarinové receptory nacházející se na žlázách a hladkém svalstvu. Muskarinové receptory patří do velké skupiny receptorů spřažených s G proteinem. Existuje celkem pět subtypů muskarinových receptorů (M1-M5), které v závislosti na typu působí excitaci nebo inhibici. V synaptické štěrbině je acetylcholin hydrolyzován acetylcholinesterasou. Právě inhibice této acetylcholinesterasy, např. pomocí pesticidů, způsobuje akumulaci acetylcholinu v synaptické štěrbině vedoucí k nadměrné stimulaci cholinoceptorů.

Muskarinové receptory a acetylcholinesterasy jsou důležité terapeutické cíle v mnoha onemocněních, jakými jsou neurodegenerativní poruchy, poruchy dolních močových cest, kardiovaskulární poruchy, nebo onemocnění dýchacích cest. V lékové terapii jsou občas používány antagonisté muskarinových receptorů, ale díky vysoce konzervované orthosterické doméně muskarinových receptorů ztrácí selektivitu vůči jednotlivým podtypům, což by mohlo být prospěšné z pohledu možných nežádoucích účinků. Vyvíjeny jsou alosterické (sekundární vazebná místa) modulátory, které působí selektivně na jednotlivé muskarinové subtypy receptorů, a které poskytují nevé možnosti ve vývoji nových léčiv. Jedním takovým alosterickým modulátorem je obidoxim, který kombinuje účinek antimuskarinový s inhibicí esterasy.

V této práci byly studovány tři typy sloučenin - silný inhibitor cholinesterasy (fysostigmin), slabý inhibitor cholinesterasy vykazující antagonismus vůči muskarinovému

receptoru (např obidoxim) a "selektivní" antagonista muskarinového M2 receptoru (methoktramin). Obidoxim má "selektivní" antagonistický profil k M2 receptoru. Cílem práce bylo zjistit funkční význam muskarinových M2/M3 receptorů ve vztahu k inhibici acetylcholinesterasy. Častý výskyt muskarinových M2 receptorů na hladkém svalstvu dýchacích cest a dolních močových cest mátlo vědce po mnoho let. Současné farmakologické přístupy využívané v této práci nám umožnily studovat a objevovat nové interakční mechanismy M2 a M3 muskarinových receptorů, které mohou mít zásadní význam pro toxicitu ve farmakoterapii využívající inhibici esterasy.

Metodika: Funkční studie byly prováděny na izolovaných orgánech (potkaní atrium a proužky močového měchýře) s použitím orgánové lázně in vitro. V in vivo experimentech byly u anestetizovaných potkanů studovány srdeční frekvence a tlak v močovém měchýři.

Klíčová zjištění: Výsledky potvrzují, že obidoxim vykazuje antimuskarinové účinky a že inhibiční profil vykazuje selektivitu k M2 receptoru. Antimuskarinový účinek je pak mnohem menší než účinek vyvolaný atropinem. Výsledky rovněž ukazují, že se inhibice esterasy a antagonismus muskarinových receptorů projeví v závislosti na koncentraci a dávce studované látky.

Dosavadní výsledky také objevují novou roli M2 muskarinových receptorů, a to že, alespoň při nízké intenzitě cholinergní stimulace, stabilizují močový měchýř způsobem málo citlivým vůči acetylcholinu. Tato zjištění bychom měli mít na paměti při podávání látek, které zvyšují cholinergní účinky současně s projevy antagonismu vůči muskarinovému M2 receptoru. V důsledku toho, při použití reaktivátorů acetylcholinesterasy, je důležité podávat oximové reaktivátory, které vykazují nižší afinitu k M2 muskarinovému receptoru.

Závěr: Tato disertační práce prokazuje, že stimulace muskarinových M2 receptorů při nízké hladině acetylcholinu v synaptické štěrbině inhibuje kontrakční odezvy vyvolané muskarinovým M3 receptorem. Toxicita reaktivátorů cholinesteras může být spojena s dříve popsaným antimuskarinovým profilem receptoru M2. Propojení muskarinového M2 a M3 receptoru by tak mohlo vyvolat protichůdný mechanismus v léčbě využívající inhibici acetylcholinesterasy, pokud budou použity reaktivátory typu obidoximu.

Klíčová slova: atrium, močový měchýř, cholinoceptory, acetylcholinesterasa, muskarinové receptory, inhibitory acetylcholinesterasy, physostigmin, obidoxim, antagonisté muskarinu, atropin, methoktramin, přeslech, agonisté muskarinu, metacholinu, acetylcholin.

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Abbreviations

NS	Nervous System
CNS	Central Nervous System
PNS	Peripheral Nervous System
ANS	Autonomic Nervous System
SNS	Sympathetic Nervous System
PSNS	Parasympathetic Nervous System
ENS	Enteric Nervous System
SoNS	Somatic nervous system
PKPD	Pharmacokinetics and Pharmacodynamics
Ach	Acetylcholine
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
GPCR	G-protein coupled receptors
IP_3	inositol-1, 4, 5-triphophate
MCh	acetyl-β-methylcholine chloride (Methacholine)
NA	Noradrenaline
OAB	Overactive Bladder
$[Ca^{2+}]I$	Calcium ions
COPD	Chronic Obstructive Pulmonary Disorders
CVS	Cardio Vascular Disorders
AD	Alzheimer's disease

OPP	organophosphate poisoning
cDNA	complementary deoxyribo nucleic acid
TM	transmembrane
CT	cross talk

Contents

1. Introduction

Muscarinic receptors regulate a number of different physiological functions in the body. In the heart, the transmitter acetylcholine lowers the rate by acting on the muscarinic receptor. The smooth muscles of airways, the gut and the urinary bladder contract in response to muscarinic receptor stimulation, while muscarinic receptor stimulation induces secretion in many glands. In addition, muscarinic receptors play important roles in the central nervous system. One area of particular interest is the role of muscarinic receptors in cognition. Thus, muscarinic receptors are important therapeutic targets in the pharmacotherapies of several diseases like neurodegenerative disorders, cardiovascular disorders, overactive bladder, chronic obstructive pulmonary disorder (COPD) and autoimmune disorders (nicotinic acetylcholine receptors- e.g. myasthenia gravis) (Clark 2012, Goodman et al 2008, Rang 2007).

The endogenous agonist acting on the muscarinic receptors is acetylcholine. Acetylcholine is a phylogenetically old neurotransmitter, which is widely distributed in the body (Amenta & Tayebati 2008). Acetylcholine is synthesized within the cell by the enzyme choline acetyltransferase catalysing choline in combination with acetyl-coenzyme A and is stored in neuronal vesicles. At stimulation a Ca^{2+}-dependent release occurs, and within the synaptic cleft, or to some minor extent within the bloodstream, a degradation of acetylcholine by acetylcholinesterases eventually occurs.

The receptors belong to G-protein coupled family receptors. Muscarinic receptors are pharmacologically subdivided into muscarinic M1, M2, M3, M4, and M5 receptors. Muscarinic receptors activate secondary messengers like phosphotidylinositol triphosphate or inhibit adenylyl cyclase to produce the cellular effects. Pharmacological investigations are unable to completely unravel the functions of muscarinic receptors subtypes due to lack of selective agonists and antagonists. Gene targeting techniques have made it possible to specify different functions of muscarinic receptor subtype (Ishii & Kurachi 2006).

To study the molecular basis of muscarinic receptor function a variety of different mutagenesis techniques have been used. Cloned muscarinic receptor cDNAs studies have thus identified the distinct receptor domains, which are individual amino acids, which participates in ligand binding affecting the G-protein coupling. The binding to the receptor is of course the prerequisite for an agonist being able to activate the cellular machinery (Brann et al 1993a, Wess 1993).

The third cytoplasmic (intracellular) loop (i3) of the receptor domains N-terminal portion defines the selectivity for different G proteins. Epitopes, which are multiple and discontinuous, contribute to their selectivity for different ligands. It is suggested out of site directed mutagenesis and covalent labelling studies, that the ligands bind at the hydrophobic core of the receptors (which is formed by multiple TransMembrane (TM) domain). For the agonist binding, in contrast to that of antagonists, amino acids having multiple hydroxyl groups enhance the binding. Aspartic acid, an amino acid, located in TransMembrane 3 binds to muscarinic ligands via the ammonium head group (Brann et al 1993b).

The TransMembrane domains (III, V, VI and VII) upper half contains hydrophilic amino acids, which forms the acetylcholine-binding domain. For the muscarinic receptor family Aspargin (TransMembrane VI Asn) residue is the characteristic e.g. Asn507 in the rat M3 receptor sequence. When transmembrane VI Asn residue is modified, it has little effect on high affinity acetylcholine binding, receptor activation or reduction in binding affinities for some subclasses of muscarinic antagonists. The i3 loop forms an amphiphilic alpha-helix (insertion mutagenesis studies). The hydrophobic side of the amphiphilic alpha-helix represents an important G protein recognition surface. Tyrosine (Tyr)254 located at the N-terminus of the i3 loop plays a key role in muscarinic receptor-induced Gq activation which was revealed by mutational analysis (Wess et al 1995).

G protein coupled receptor kinases phosphorylates agonist bound to the muscarinic receptors. These kinases initiate the receptor desensitisation through uncoupling from G proteins, down regulation (receptor breakdown) and receptor internalisation (Haga 2013).

In the current thesis the effects of cholinesterase inhibitors and muscarinic receptor antagonists have been studied in the cardiovascular system (the heart rate) and in the lower urinary tract (bladder contraction). While the heart expresses inhibitory muscarinic M2 receptors, the detrusor exhibit contractile muscarinic M3 receptors as well as inhibitory muscarinic M2 receptors (Andersson 2011). However, inhibitory muscarinic receptors are present both presynaptically and postsynaptically. Presynaptic muscarinic receptors modulate the release of other neurotransmitters (Levey et al 1991, Tobin 1995, Tobin 1998, Tobin & Sjogren 1995). This indicates the intricate complexity of how the cholinergic systems result in functional responses.

Acetylcholinesterase (AcetylcholineE: EC 3.1.1.7) is a serine protease, which hydrolyses acetylcholine neurotransmitter into acetate and choline. In the current thesis project an

acetylcholine esterases ligand have been used in order to elucidate muscarinic receptor interactions.

Physostigmine (Eserine) is an alkaloid present in physostigma venenosum (fabaceous plant) (Percy L. Julian & Pikl 1935). The structure of physostigmine is 1,2,3,3a,8, 8a,-hexahydro-1,3a,8-trimethyl-pyrrolo[2,3-b]indo-5-ol-methylcarbamate. It is a carbamate reversible inhibitor of acetylcholinesterase (Scheindlin 2010, Traub et al 2002). Physostigmine acts on acetylcholine receptor complexes and acetylcholine gated cation channels. Physostigmine is non-ionic, tertiary amine which is a lipid soluble compound. Physostigmine is clinically used to treat glaucoma, myasthenia gravis etc. It has narrow therapeutic window (Mach et al 2004, Meshulam et al 2001, Somani & Dube 1989). As a peudosubstrate or competitor physostigmine interacts with acetylcholinesterase and prevents endogenous acetylcholine from reacting with the acetylcholinesterase enzyme and thus retain the local concentration of acetylcholine at the synapse (Triggle. et al 1998).

Obidoxime (1,3-bis (4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride), a well-known bis-pyridinium reactivator, is often the preferred antidote of organophosphorus poisoning caused by pesticides and tabun. Obidoxime is considered to be an allosteric modulator (antagonist) of the muscarinic M2 receptor. It inhibits the binding of [3H] acetylcholine and slows the rate of dissociation of [3H] acetylcholine in a concentration-dependent manner. Obidoxime has been reported to be a competitive ligand at the common allosteric site and described as negative allosteric modulator. The antagonistic activity of obidoxime could be of important clinically (Soukup et al 2010b).

Methoctramine (N, N'-bis [6-[[(2-methoxybenzyl)-amino] hexyl]-1, 8-octane] diamine) is a polymethylene tetraamine. Methoctramine is a cardioselective M2 selective competitive antagonist of muscarinic receptors (Wess et al 1988). It exhibits allosteric properties at high concentrations. Methoctramine shows selectivity to M(2) receptors because of interactions with both the orthosteric and the allosteric binding sites located between the second and third extracellular loops (Jakubik et al 2014). However, methoctramine may also acts as an antagonist on nicotinic receptors in the airways (Watson et al 1992). This is major tool for the functional studies of muscarinic receptors subtype M2 because of its relative specificity. It may, however, show some cytotoxicity effects at high concentrations (Zini et al 2009).

The functional significance of muscarinic M2 receptors in the state of acetylcholinesterase inhibition, elucidating muscarinic M2 and M3 receptor interactions may be of great clinical

significance due to the unravelled mechanisms of toxicity of reactivators. And this thesis addresses one issue that has been unexplained for many years – namely the significance of the large number of muscarinic M2 receptors that occurs on the detrusor cell. What makes this phenomenon of particular interest is that the situation in the respiratory tract shows a pronounced resemblance with that in the urinary tract. This seems to be of particular interest in view of the toxicity of reactivators causing breathing arrest.

When the work on the current project started, it was known that the acetylcholine esterase reactivator, obidoxime, could act as an allosteric modulator on cardiac muscarinic receptors (Ellis & Seidenberg 1992, Jakubík & El-Fakahany 2010). In the first paper (Soukup et al 2010b) of this thesis, obidoxime was shown to exert different functional antimuscarinic effects in the heart and the urinary bladder. These effects have subsequently been further elucidated in a thesis from the Department of Toxicology, Faculty of Military Health Sciences, University of Defence (Soukup 2011).

In the next paper in this thesis (Killi et al 2014), we were able to identify a new principle by which muscarinic M2 and M3 receptors interact. We suggest that this principle explains some of the obidoxime toxic effects.

In the third paper of this thesis (Killi et al 2015), the functional significance of antimuscarinic drug effects, such as that of obidoxime, are discussed in the perspective of different organ functions.

2. THEORETICAL PART

2.1. Nervous System

The normal physiological functions of the body helps to maintain the dynamic balance of the organ systems which is necessary for a healthy life. The organ systems in the body are regulated by the nerves and its biochemical mediators which are released in response to action potentials (Clark 2012, Goodman et al 2011, Rang 2007).

The central nervous system (CNS) and the peripheral nervous system (PNS) constitutes the nervous system anatomically in the whole human body. The CNS constitutes the brain and the spinal cord and the PNS includes afferent nerves and efferent nerves. From the CNS to the parts of the body, other than the brain and the spinal cord, the autonomic nervous system (ANS) takes the involuntary signals (Clark 2012, Goodman et al 2011, Rang 2007)

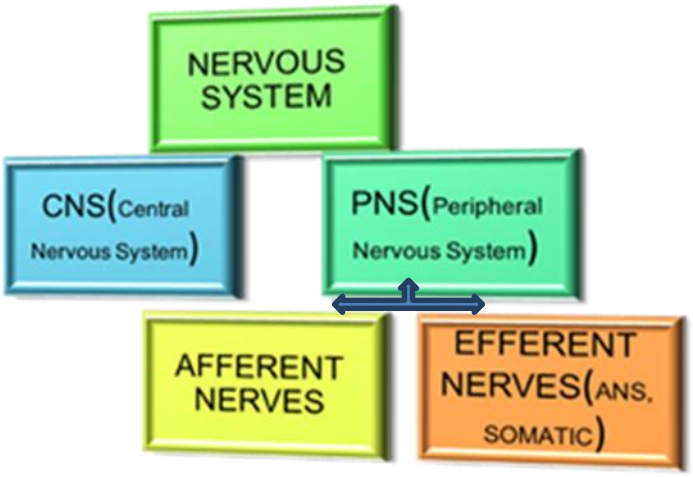


Figure 1. Schematic diagram of nervous system. The peripheral nervous system classified into afferent nerves and efferent nerves. Efferent nervous system constitutes both autonomic nervous system and somatic nervous system.

2.2 Autonomic Nervous System

The autonomic functions are regulated by the ANS which is widely distributed throughout the body. Alternative terms for ANS are the *visceral*, *vegetative*, or *involuntary nervous system*. The ANS consist of nerves (neurons), ganglia (synapses), and plexuses (branching network of axons outside of the central nervous system) in the periphery which innervates and regulates **the heart**, blood vessels, glands, other visceral organs, and **smooth muscle** in various tissues (urinary bladder) (Bennett 1997, Clark 2012, Goodman et al 2011, Rang 2007).

The ANS constitutes the sympathetic (SNS) and parasympathetic (PSNS) and the enteric nervous system (ENS). The ENS, because of its many neurons, is called the second brain (Gershon 1999) and it controls the gastrointestinal tract (Clark 2012, Goodman et al 2011, Rang 2007).

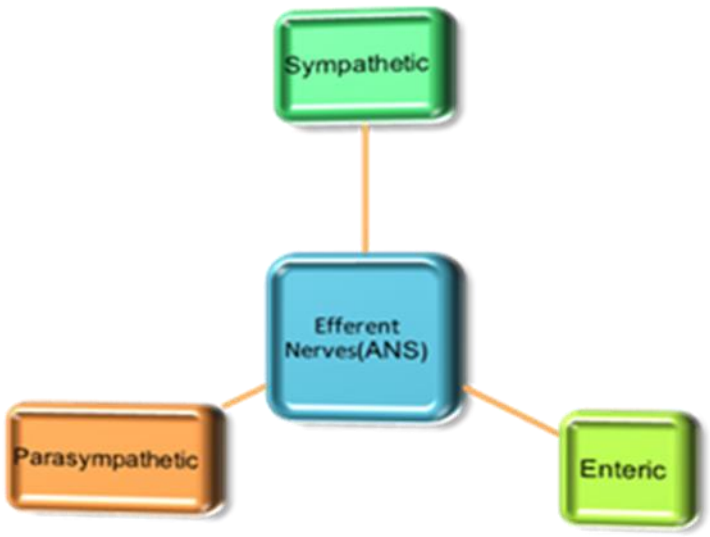


Figure 2. Schematic representation of Autonomic nervous system which includes sympathetic nervous system, parasympathetic nervous system and enteric nervous system.

The PNS includes the ANS and the somatic nervous system (SoNS). There is an anatomical difference between the autonomic efferent nervous system and the somatic efferent nervous system. In the ANS, involuntary regulation, two neurons (basic units of nervous system) are arranged in a series while in the somatic efferent nervous system, voluntary

regulation, the motor neuron directly connects to the skeletal muscle fibre (Clark 2012, Goodman et al 2011, Rang 2007).

2.3. Autonomic Ganglia

Autonomic ganglion consists of synapses, which contains nerve endings of the preganglionic fibres and the cell bodies of postganglionic neurons. The coupling within the ANS occurs between the preganglionic neurons (nerve fibres of neuron) and postganglionic neurons (cell bodies of neuron) in autonomic ganglia. Autonomic ganglia could be a SNS. In the SNS autonomic ganglia are present, often located close to the spine, whereas discrete ganglia are present in the PSNS i.e. postganglionic neuron cells are found in the target organs (Clark 2012, Goodman et al 2011, Rang 2007).

2.4. Neurotransmitters

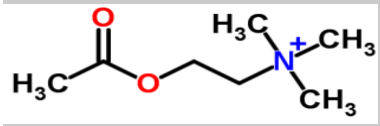
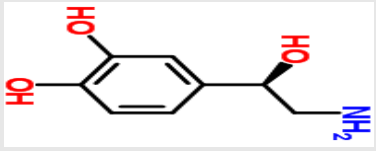
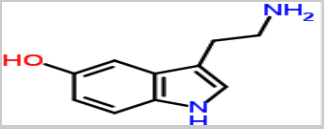
Neurotransmitters are the biochemical compounds, which conducts the signals in the nervous system. The major neurotransmitters in the ANS are acetylcholine and noradrenaline (NA) (Clark 2012, Goodman et al 2011, Lodish 2008, Rang 2007).

All the preganglionic neurons are cholinergic. On the postganglionic nerve cells excitatory muscarinic receptors exists. All the postganglionic parasympathetic neurons are also cholinergic since the acetylcholine stimulates the muscarinic receptors present on the target organs. Sympathetic postganglionic neurons are mostly noradrenergic, except for some e.g. sweat glands which are cholinergic. Nicotinic acetylcholine receptors participate in ganglionic neurotransmission (Andersson & Arner 2004, Andersson & Wein 2004, Clark 2012, Goodman et al 2011, Rang 2007).

Neurotransmitters in the ANS other than noradrenaline and acetylcholine are called Non-Adrenergic Non-Cholinergic (NANC) transmitters. Examples of NANC transmitters are nitric oxide (NO), vasoactive intestinal peptide (VIP), Adenosine Triphosphate (ATP), neuropeptide Y, and 5-hydroxytryptamine (serotonin) (Bennett 1997, Clark 2012, Goodman et al 2011, Rang 2007).

Acetylcholine is released from the presynaptic nerve terminal into cholinergic synapses. Within the synapse, it is hydrolysed by acetylcholinesterase into choline and acetyl coenzyme A. In the synapse the choline (obtained from the acetylcholine hydrolysis) reuptake, rate limiting step in acetylcholine synthesis, was done by the high affinity choline transporter. Choline is to be given from outside e.g. through diet, since neurons does not have choline on its own(Amenta & Tayebati 2008). By the influence of choline acetyltransferase, acetylcholine is synthesized excessively (Clark 2012, Goodman et al 2011, Rang 2007).

Table 1. Neurotransmitters chart with chemical structure, mode of action, and their location adapted from PubMed chem.

Neuro-transmitter	Chemical Structure	Mechanism of Action	Location
Acetylcholine		Binding to Ion-gated channels and/or Through G-proteins and secondary messengers	In the CNS, autonomic ganglia, Neuro-muscular junctions, Para-sympathetic effector junctions, a subset of sympathetic effector junctions.
Norepinephrine		Through G-proteins and secondary messengers	Cerebral Cortex, Brain Stem, Spinal Cord, Hypothalamus, Neuromuscular Junctions and Neuroglandular Junctions (ANS)
Serotonin		Through G-proteins and secondary messengers	Retina, Limbic-System, Hypothalamus, Cerebellum, and Spinal Cord.

2.5. Receptors

2.5.1. Ligand-gated ion channels

Ligand-gated ion channels, combines the receptor and ion channel functions in a single molecule (Purves & Williams 2001), are also known as ionotropic receptors, which are the large group of neurotransmitter receptors. They are involved in the fast synaptic transmission, which normally occurs in milliseconds at somatic neuromuscular junctions. These receptors have a binding site (extra cellular domain) and an aqueous ion channel (multisubunit membrane spanning proteins). The ion channel is formed by four or five heteromeric subunits, as shown in the below diagram (Figure 3). Examples for this type of receptors are the nicotinic acetylcholine receptors (nAChR), glutamate receptors of the NMDA, gamma amino butyric acid (GABA) type A receptors ($GABA_A$), AMPA and kinate receptors and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors (Clark 2012, Goodman et al 2011, Rang 2007).

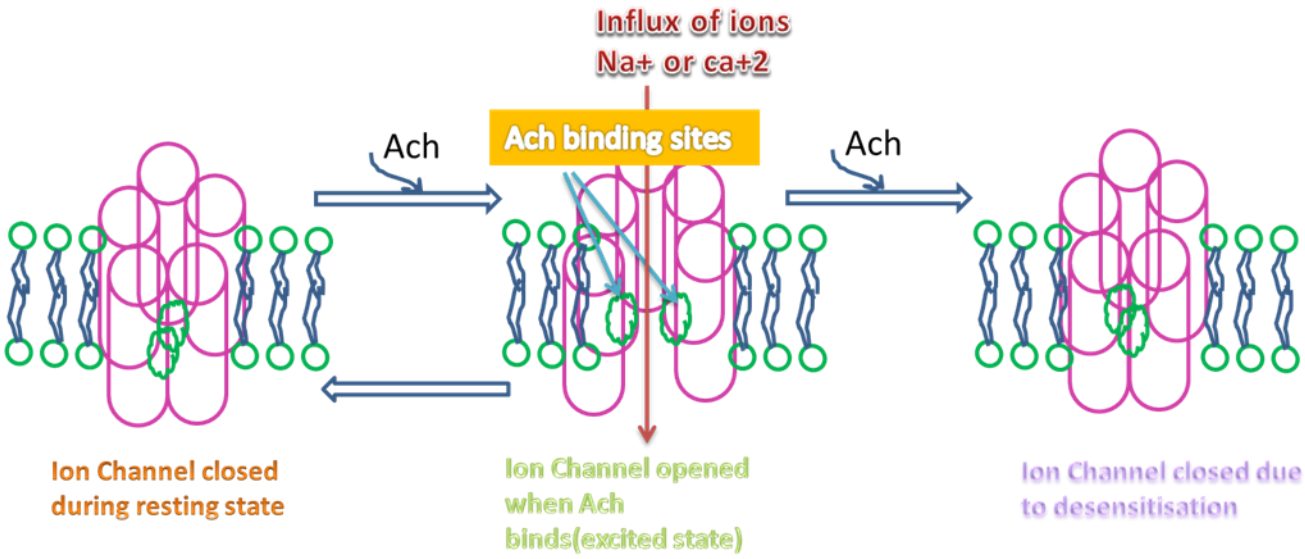


Figure 3. Ligand-gated ion channels Influx of ions (e.g. Na^+ and Ca^{+2}) by opening of ion channels when acetylcholine binds and resensitisation when acetylcholine is broken down and desensitisation when acetylcholine is accumulated (adopted and modified from (Clark 2012, Goodman et al 2011, Rang 2007)).

2.5.2. G protein-coupled receptors (GPCRs)

GPCRs are also known as metabotropic receptors or 7-transmembrane (7-TM or heptahelical) receptors. This contains single polypeptide chain. These polypeptide chain spans as seven transmembrane α-helices with N-terminal (extracellular) and C-terminal (intracellular) domains which is the characteristic feature of GPCRs. These contain G protein which consist of three subunits called α, β, γ. Normally α subunit is the active subunit but occasionally the βγ subunit is the activator species. Examples of the GPCRs can be muscarinic receptors, opiate receptors, purine receptors, chemokine, neuropeptide receptors, and adrenoceptors (Clark 2012, Goodman et al 2011, Rang 2007). They are membrane receptors that are coupled to intracellular effector systems via a G protein (see in 2.6 section on page 26).

2.5.3. Kinase-linked and related receptors

Kinase-linked and related receptors are a large, heterogeneous group that often responds to protein mediators. These are also called membrane receptors like GPCRs. The extracellular ligand-binding domain and an intracellular domain are linked together by a single transmembrane helix. The intracellular domain is enzymatic in nature e.g. protein kinase or guanylyl cyclase activity, but cytokine receptors intracellular domain lacks the enzymatic activity.

Signal transductions comprises two steps called dimerization and phosphorylation. On ligand binding dimerization occurs. The two intracellular kinase domains associate for autophosphorylation. Thus phosphorylated residues acts a high affinity binding site for various proteins to perform various cell functions within the cell. These receptors indirectly involved in gene regulation by Ras/Raf/Mitogen-Activated Protein (MAP) kinase pathway and Jak/Stat pathway.

Kinase-linked and related receptors are the receptors for insulin, for various cytokines and growth factors; Guanylyl cyclase (hormone receptors-exhibit structural similarity but differ in their transduction mechanisms) is the receptor for atrial natriuretic factor (ANF) (Clark 2012, Goodman et al 2011, Rang 2007).

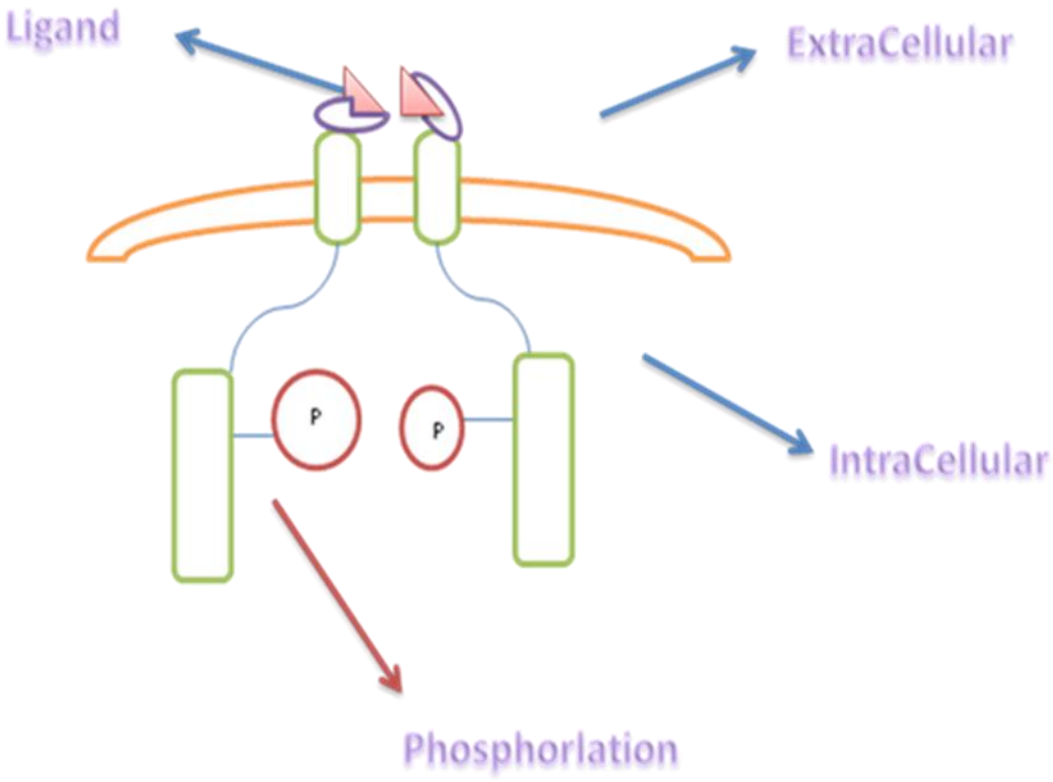


Figure 4. Kinase-linked and related receptors undergo dimerization (ligand induced) and then activated. Each kinase domain is activated by the other because of phosphorylation. Adopted and modified from (Kuriyan & Eisenberg 2007).

2.5.4. Nuclear receptors

Nuclear receptors are the receptors that regulate gene transcription. They may be either cytosolic or nuclear. Nuclear receptors include two subtypes homodimers and heterodimers.

Some of them are homodimers, which are present in the cytosol. In the presence of a ligand (which is endocrine in nature e.g. steroid hormones) they move to the nucleus and the other is receptors present in the nucleus and form heterodimers with the retinoid X receptor in the presence of ligands (which are lipids usually e.g. the fatty acids). There also exists a third subtype (mixture of above two subtypes) that participates in endocrine signalling by heterodimerisation with retinoid X receptor (e.g. the thyroid hormone).

Structure of the nuclear receptors contains N-terminal domain, core domain, hinge region, C-terminal domain. Heterogenic N-terminal domain contains activation function 1 (AF1) site. Highly conserved core domain, contains zinc fingers, helps to recognise and bind DNA. Highly flexible hinge region helps to dimerise with other nuclear receptors and the C-terminal possess highly conserved AF2 (ligand binding region) which is specific to each receptor class. In gene promoters the liganded receptor complexes bind to hormone response elements by recruiting co-activator or co-repressor factors, there by modulation of gene transcription was initialised.

Nuclear receptors include the' orphan receptors.' Orphan receptors' are those with no specific ligands e.g. RXR receptor. They include receptors for steroid hormones, thyroid hormone and other agents such as retinoic acid and vitamin D. E.g. Glucocorticoid receptors (GR), oestrogen receptor (OR), peroxisome proliferator receptor (PPAR). Receptors of this type also recognise many foreign molecules (xenobiotics), inducing the expression of enzymes that metabolise them (Clark 2012, Goodman et al 2011, Rang 2007).

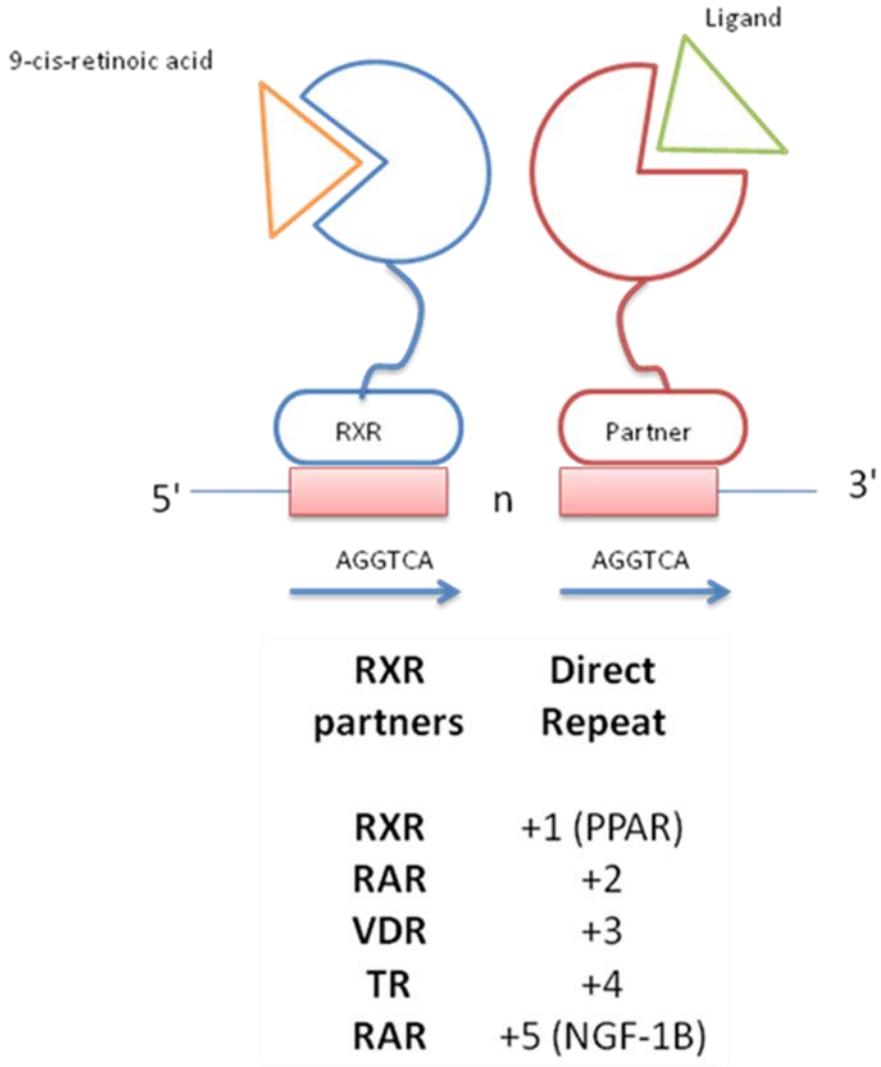


Figure 5. Non-steroidal **Nuclear receptors** function as heterodimers, with RXR (Retinoid X Receptor), or as homodimers also. RXR identifies a direct repeat hexad AGGTCA. This hexad separated by one to five nucleotides (n). n denotes thyroid-hormone receptor (TR), peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor (RAR), Vitamin D receptor (VDR), NGF-1B (Nuclear growth factor inducible receptor) (Tata 2002).

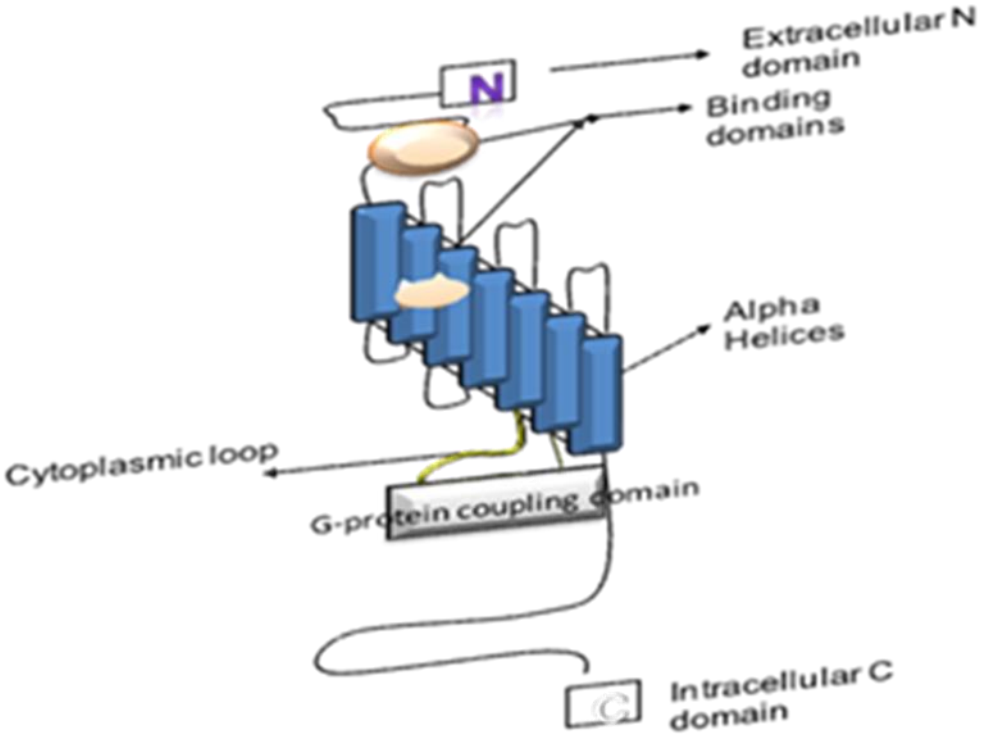
2.6. Molecular structure of G Protein Coupled Receptors (GPCRs) and their signal transduction.

A large number of signalling molecules, such as neurotransmitters and hormones, couple to the interior of the cell via G proteins, and consequently this family of receptors is named G-protein coupled receptor. Often the acronym GPCR is used in the literature.

The seven transmembrane-spanning polypeptide that forms the G-protein coupled receptor, interacts via conformation changes with the G-proteins. The G-proteins are heterotrimeric, GTP-binding proteins. The G-protein regulation of a functional response depends on its effect on intracellular systems, which was finally demonstrated in the early 90s in experiments employing cell lines expressing cloned receptors (Iismaa & Shine 1992).

The importance of G-protein coupled receptors for physiological effects is obvious. However, they may also have key roles in disease – cholera is a well-known example in which toxins affect the G-protein. Since this group of receptors are very common, it follows that a large number of pharmaceuticals target GPCRs of one kind or another. One example of receptors belonging to this receptor family is the muscarinic receptors, which this thesis very much focuses on. Other examples are the adrenoceptors, the dopamine receptors, 5-HT receptors and the opioid receptors. All are very important targets in pharmacotherapy.

The receptors of this family may comprise of single polypeptide chains up to 1100 residues. The polypeptide has an extracellular N-terminal part and an intracellular C-terminal part because of the amino acid building units. The passage in and out of the cell membrane forms loops internally (and externally). The largest loop usually interacts with the G-protein (Clark 2012, Goodman et al 2011, Jackson 1991, Rang 2007).



Structure of GPCRs

Figure 6. **Structure of GPCRs**- 3D view of 7 α-helices with 2-binding domains and G-protein coupling domain

On activation, i.e., the binding of an agonist, the conformation change causes, under the influence of GDP, binding dissociations of the α-, β- and γ-constituents of the G-protein. GDP is important for the specific receptor-effector recognition. Depending on the type of G-protein, it may induce inhibition or excitation.

The Gα subunit regulates upon activation via GTP an enzyme or ion channel. Also the Gβγ subunits disassociates from the Gα subunit. Until the GTP is hydrolysed to GDP, through GTPase, the G protein will be in an active state.

βγ subunits may act synergistically or act directly via K+ (GIRK) channels). Eventually, the βγ subunits and the GDP-liganded Gα subunit re-combine.

Unlike ionotropic receptors, G protein coupled receptor responses last for several seconds to minutes. Enzymes (effectors) affected by G protein are adenylyl cyclase, phospholipase C, phosphodiesterases, and plasma membrane ion channels selective for Ca2+ and K+. These effectors then change the concentrations of second messengers (e.g. cyclic adenosine monophosphate (cAMP)). These second messengers are responsible for further actions within the cell.

G protein coupled receptors are group into three distinct types. The largest group (rhodopsin family) consists of most monoamine, neuropeptide and chemokine receptors. The other group of receptors (secretin/glucagon receptor family) are receptors for some peptides like calcitonin and glucagon. The smallest group include for instance GABAergic receptors (metabotropic glutamate receptor/ Gamma Amino Butyric Acid (GABA)/ calcium sensor family).

Within the family they are having similar structures, but in between the families they have different length of extracellular N terminal domain and the location of agonist binding domain, and also in some other aspects. Second messengers like cAMP, inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol, plays a key role in amplifying and conducting signals from the G protein–coupled receptors.

G proteins (G_s) with α-GTP subunits regulate adenylyl cyclase activation which produces cAMP. cAMP regulates the protein phosphorylation. G_q protein (for nor epinephrine) activates Phospholipase Cb (PLCb). Phospholipase Cb is a membrane-bound enzyme. It hydrolyses phosphatidylinositol-4, 5-bisphosphate to produce IP3 and the lipid, diacylglycerol. Phosphatidylinositol-4, 5-bisphosphate is a membrane phospholipid. Endoplasmic reticulum

contains IP3-sensitive Ca^{2+} stores. When IP3 binds to receptors then the Ca^{2+} releasing channels opens to release Ca^{2+} ions.

G protein also activates guanylyl cyclase, to produce cyclic guanosine monophosphate (cGMP). cGMP is the fourth second messenger. cGMP signalling is important to relax the muscle in the intestinal mucosa and vascular smooth muscle. E.g. Sildenafil interferes with phosphodiesterases and causes vasodilation (Clark 2012, Goodman et al 2011, Rang 2007).

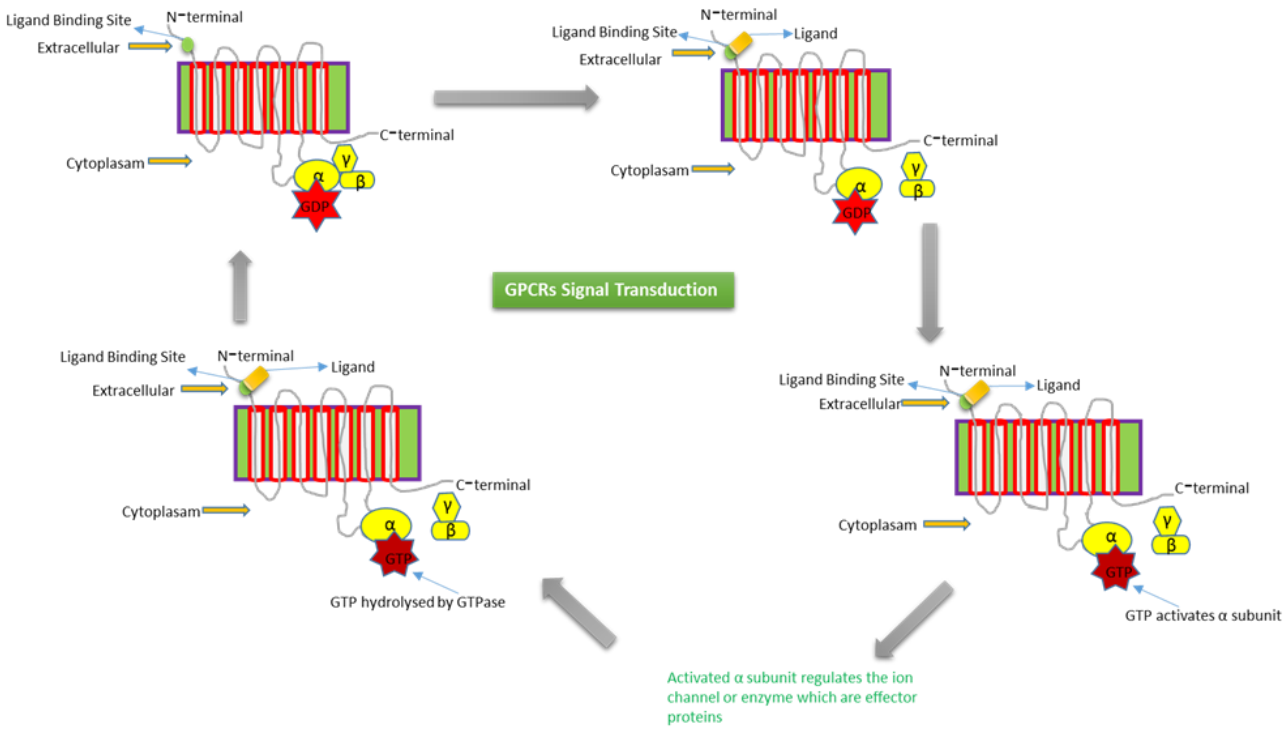


Figure 7. GPCRs Signal Transduction- Ligand binding to receptor activates G protein which regulates an enzyme or ion channel. Enzyme effects the second messengers to produce cellular effects whereas ion channels produce directly the cellular effects.

2.7. Cholinergic System

The cholinergic system comprises the nerve cell containing metabolic enzymes, neurotransmitters, cholinoceptors and their signalling pathways. Acetylcholine is one of the common neurotransmitter in the nervous systems. Cholinergic neurotransmission is conducted by the involvement of acetylcholine acting on muscarinic and nicotinic receptors, which are the therapeutic targets in diseases like cardiovascular diseases, neurodegenerative disorders, rheumatoid arthritis, over active bladder (OAB), an autoimmune disease like Sjögren's

syndrome (pSS), and also in organophosphate poisonings (Clark 2012, Goodman et al 2011, Rang 2007).

2.8. Cholinergic Transmission

Acetylcholine is formed by choline acetyltransferase within the nerve cell cytoplasm. The transmitter is then packed into synaptic vesicles (acetylcholine concentration is very high) through a carrier-mediated transport. The action potential reaching the nerve terminal causes acetylcholine to be released into the synaptic cleft by exocytosis (Ca^{2+} mediated). In the synaptic cleft acetylcholine binds to the receptors on postsynaptic cell. The breakdown of acetylcholine is a hydrolysation by acetylcholinesterase in the synapse into choline and acetate. The hydrolysed choline will be reuptaken by the choline transporter into the presynaptic nerve cell. The reuptake of choline is the rate-limiting step for acetylcholine synthesis.

One nerve impulse reaching the presynaptic terminal of nerve cell releases around 100-500 vesicles at the neuromuscular junction. At the other side of the synaptic cleft, acetylcholine produces endplate potentials when bound to the nicotinic receptors. The receptor activation opens cation channels. The muscarinic receptors neurotransmission is slower. Acetylcholinesterase inhibition, receptor antagonism, choline reuptake inhibition, acetylcholine release inhibition are the major mechanisms of pharmacological interventions (Clark 2012, Goodman et al 2011, Rang 2007).

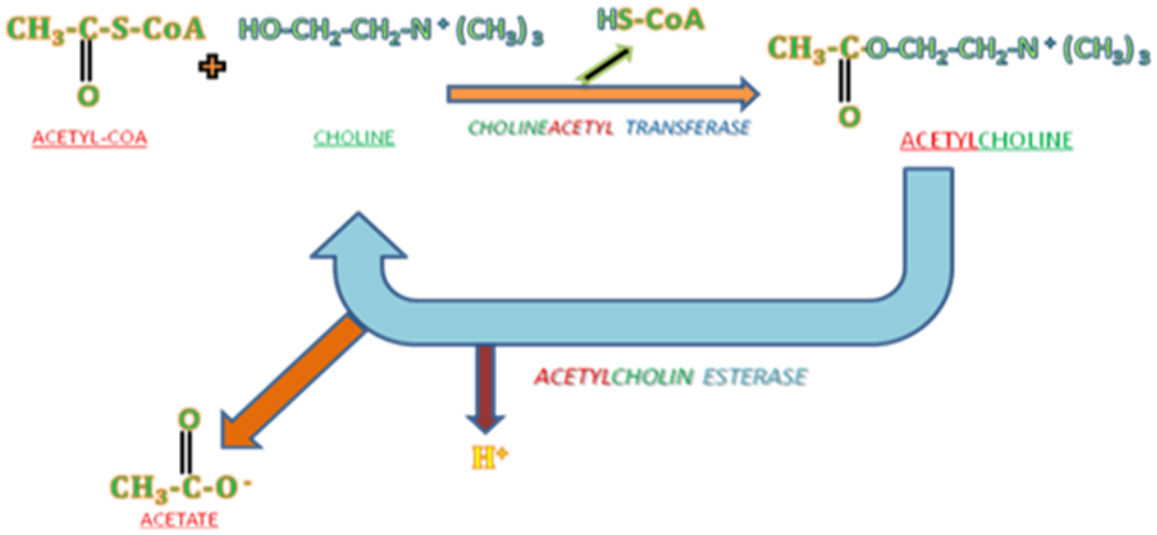


Figure 8. Acetylcholine synthesis and metabolism- Acetylcholine synthesis includes acetyl-coA and choline in the presence of enzyme called choline acetyl transferase and acetylcholine hydrolysis regulated by acetylcholine esterase in the reuptake of choline to vesicles.

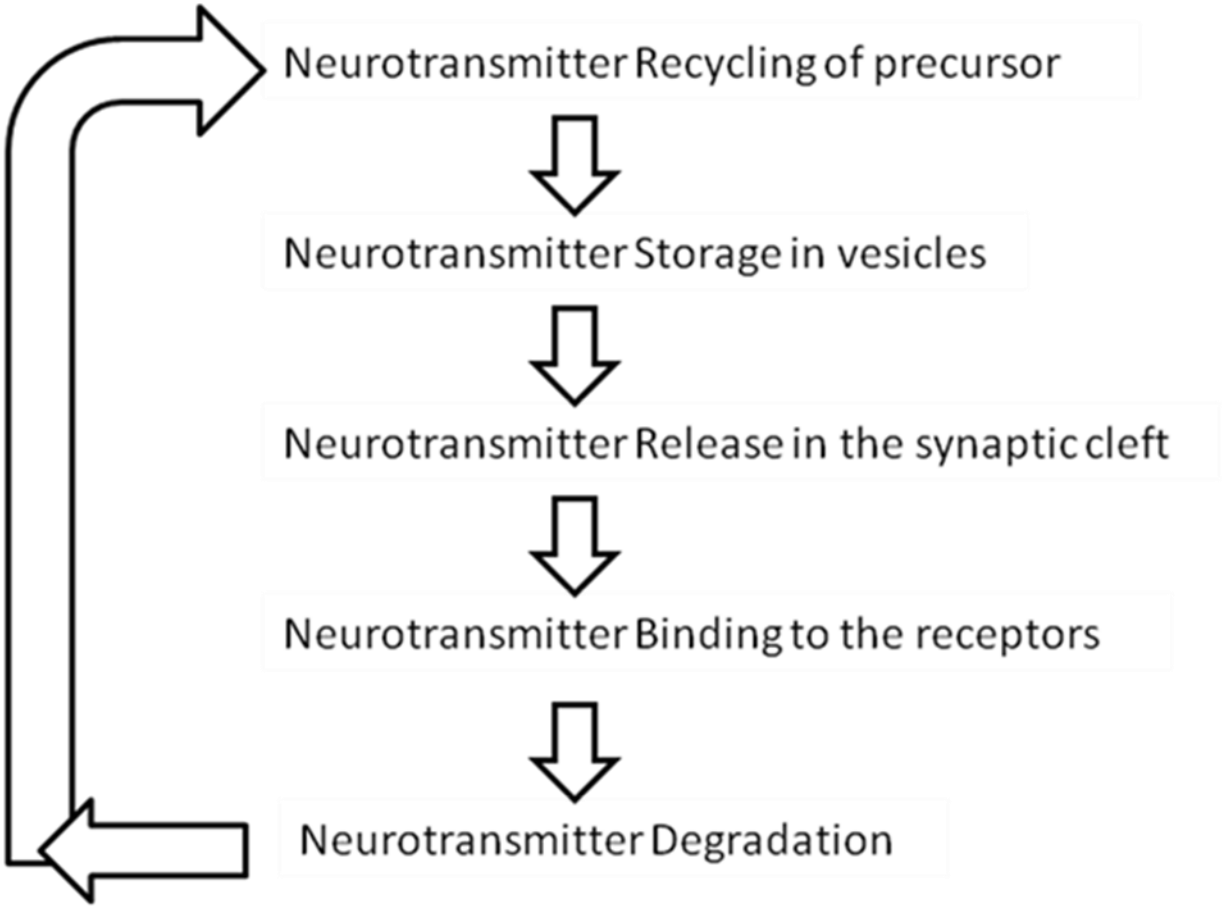
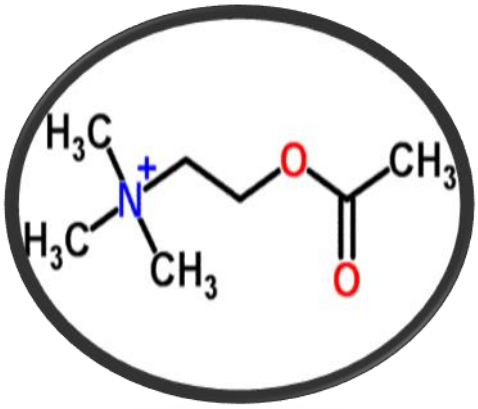


Figure 9. Cholinergic Neurotransmission- Cyclic process includes neurotransmitter synthesis, storage, release, binding to receptors, degradation, recycling of precursor.

2.9. Acetylcholine

Acetylcholine is the major neurotransmitter in the nervous system, which is an ester of acetic acid and choline. Depending on the type of receptor it interacts, acetylcholine produces either excitatory or inhibitory responses. In the nerve cell cytoplasm the acetyl coenzyme A, which produced in krebs cycle and fatty acid oxidation, combines with choline in presence of acetylcholine transferase to form acetylcholine. This may be targeted in neurological disorders and cardiovascular disorders, overactive bladder, Sjögren's syndrome (Clark 2012, Goodman et al 2011, Rang 2007)



(Chem. Spider Synthetic Page)

Figure 10. Structure of Acetylcholine

2.10. AcetylCholinesterase Enzyme

Acetylcholinesterase enzyme hydrolyses acetylcholine at the synaptic cleft (some extent in plasma). It contains catalytic globular subunits, which are linked to glycolipids or collagen like proteins. To the cell membrane/basement membrane they are chain like a bunch of balloons. It exerts non- catalytic functions in the brain, which are not well studied. Much physiology of

acetylcholinesterase enzyme is not so far elucidated very well. So this is the interesting research area. Acetylcholinesterase increases the amyloid protein fibrils formation which are toxic and linked to alzheimer's disease pathology of. Understanding the acetylcholinesterase enzyme physiology is required to find novels therapies to treat neurodegenerative disorders and organophosphate poisonings (Clark 2012, Goodman et al 2011, Rang 2007).

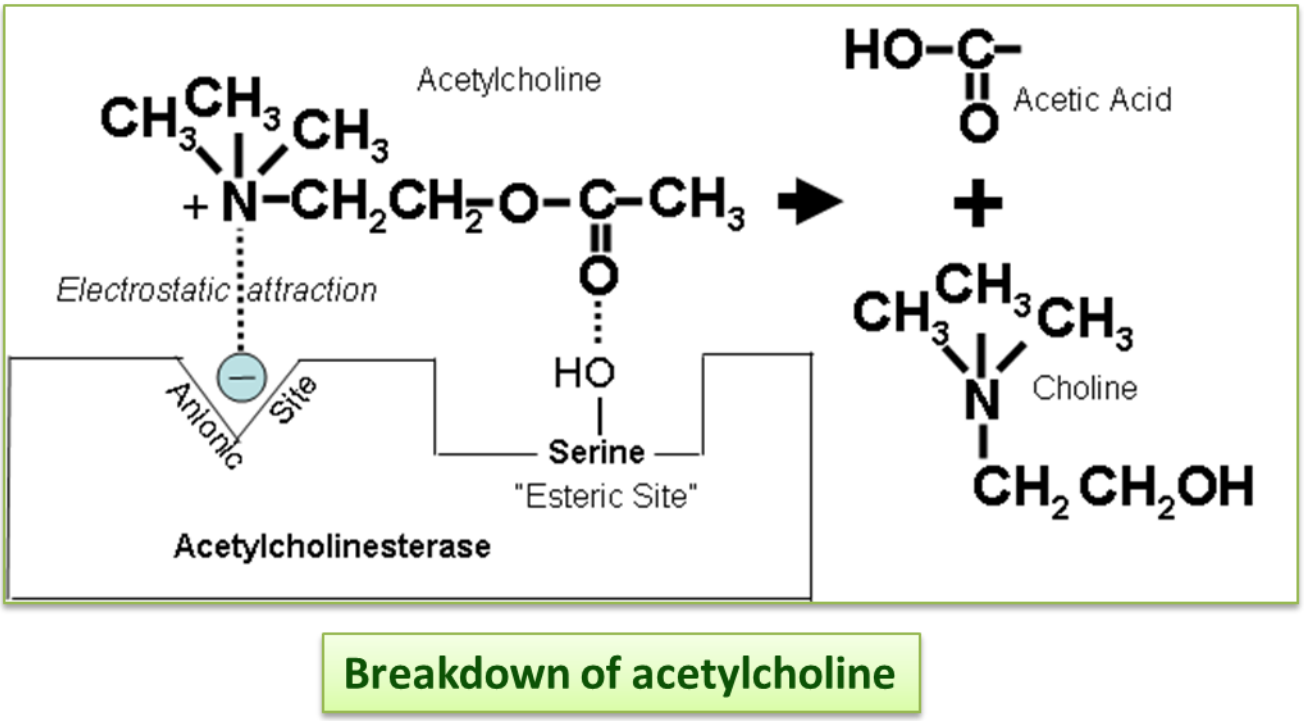


Figure 11. Acetylcholinesterase and breakdown of Acetylcholine- Acetylcholine when binds with AcetylcholineE the nitrile group of acetylcholine binds at anionic site where as C=O binds the serine hydroxyl group which is esteric site. Then acetylcholine breaks down into choline, which is reuptaken by choline transporters (ChT) into neurone for acetylcholine synthesis.

2.11. Cholinergic Receptors

The cholinergic receptor name itself defines the physiological function regulated mainly by the chemical messenger called acetylcholine. The receptors involved are muscarinic receptors and nicotinic receptors.

Acetylcholine receptors are nicotinic receptors and muscarinic receptors. Nicotinic receptors are fast excitatory receptors. They mediate fast synaptic transmission since they are directly coupled to cation channels. They are present at the various sites in the central nervous

system, autonomic ganglia and at the neuromuscular junction. Pharmacology and structure of neuronal and muscle nicotinic receptors are different. Muscarinic receptors and nicotinic receptors occur both presynaptically as well as postsynaptically. Presynaptic receptors act as autoreceptors and they regulate the neurotransmitter release (Clark 2012, Goodman et al 2011, Rang 2007).

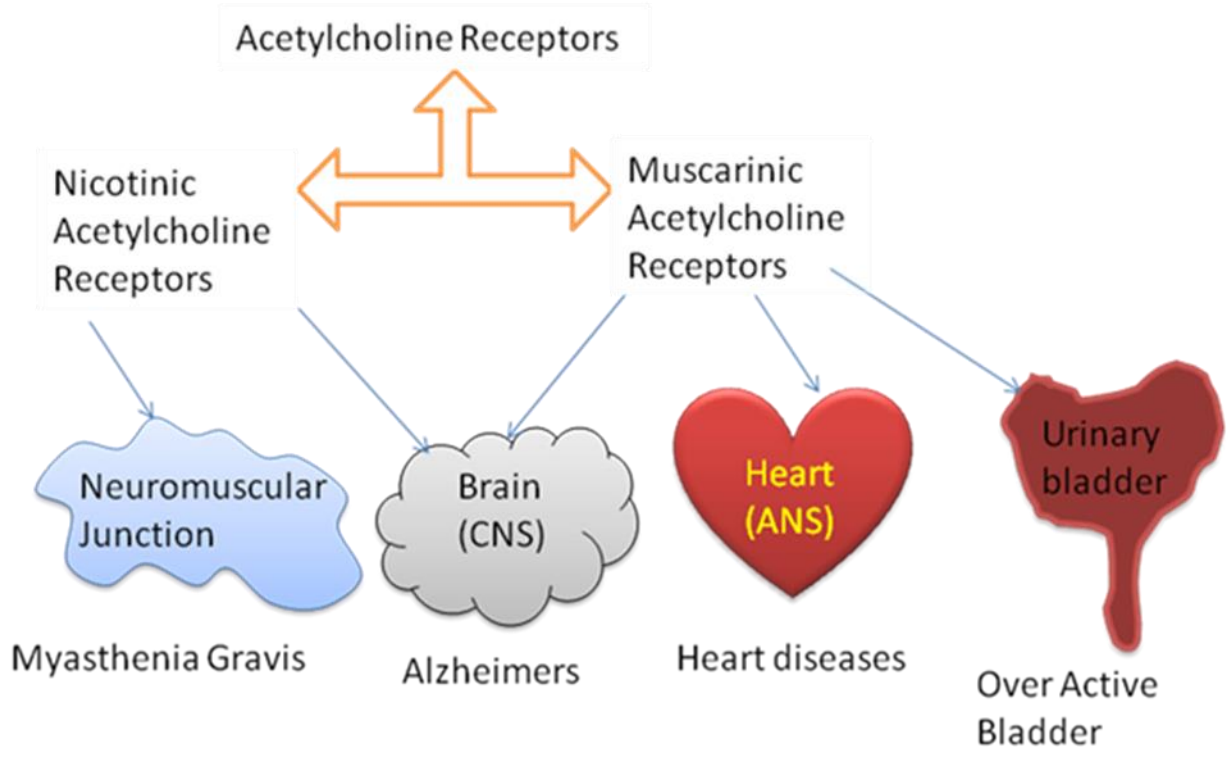


Figure 12. Cholinoceptors and their Innervation- Cholinoceptors innervate all over the body in the form of muscarinic receptors and nicotinic receptors.

2.12. Muscarinic Receptors

Muscarinic receptors subtypes have been pharmacologically characterized based on the molecular and pharmacological criteria. Muscarinic receptors are metabotropic 7-transmembrane–spanning (heptahelical) G protein (guanidine nucleotide glycoprotein) coupled receptors. Muscarinic receptors include five subclasses M1, M2, M3, M4 and M5 (Caulfield 1993, Caulfield & Birdsall 1998, Scarpero & Dmochowski 2003).

The muscarinic M1, M3 and M5 receptors are excitatory receptors, and they couple with G_q (PTX-insensitive) to activate the inositol phosphate pathway. The muscarinic M2 and M4

receptors, on the other hand, mediate inhibitory effects by coupling with G_i (PTX-sensitive) and thereby inhibiting adenylyl cyclase and thus reducing intracellular cAMP (Yokotani *et al.*, 1993; Caulfield *et al.*, 1998). More or less all muscarinic receptor subtypes have been suggested to be involved in the presynaptic modulation of neurotransmitter release in both central and peripheral neurons (Yang et al 2006).

In guinea pig carotid arteries, M_1-receptor subtype mediates inhibition of norepinephrine release (Casado et al 1992). The presynaptic M_2-receptor subtype has been considered to frequently mediate muscarinic modulation of neurotransmitter release in perivascular nerves (Liu & Lee 1999, Shen & Mitchelson 1994). The M3-receptor subtype has been shown to mediate inhibition of acetylcholine and norepinephrine release in bovine cerebral arteries (Ferrer et al 1992, Toda et al 1997). In case of the rabbit ear arteries, the prejunctional muscarinic receptors (other than the M1, M2, or M3 subtype) mediate inhibition of NE release. (Alonso et al 1991, Darroch et al 1992).

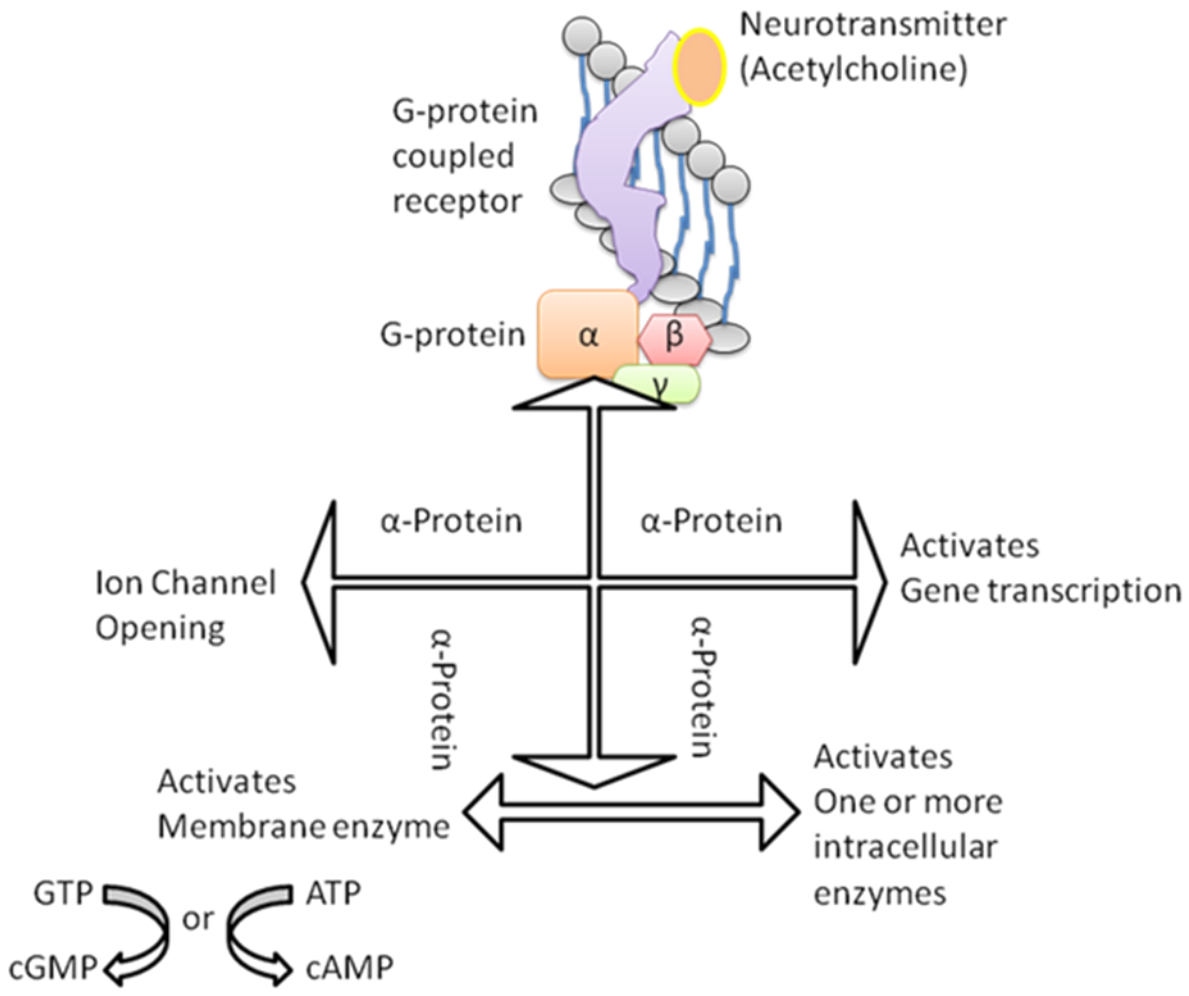


Figure 13. Nicotinic and muscarinic acetylcholine G protein coupled receptors and its signal transduction cascades.

2.12.1. Muscarinic M1 Receptors

The muscarinic receptors occur in the brain also. The most frequent muscarinic receptor subtype in the cortex, hippocampus, and striatum is the M1 subtype. Muscarinic M1 receptors are involved in cognitive processes and memory through mediating acetylcholine-induced MAP kinase activation. Lack of muscarinic M1 receptors causes increased locomotor activity because of the raise in the dopamine levels (Gerber et al 2001, Hamilton et al 1997, Hamilton & Nathanson 2001, Levey 1993, van Koppen & Kaiser 2003, Yamada et al 2001).

Muscarinic agonist depolarization of rat isolated superior cervical ganglion is mediated by M1 receptors (Brown et al 1980). This is probably the result of inhibition of opening of the voltage-gated M-type K^+ channels in these neurons (Bernheim et al 1992, Marrion et al 1989) although M1 receptors can modulate other conductance which could contribute to the depolarizing response (e.g., the protein kinase C-dependent Cl2 current described by (Marsh et al 1995).

M1 muscarinic receptor stimulates phospholipase C, and increases Ca^{2+} currents in pertussis toxin-treated guinea-pig and rat ventricular myocytes(Sharma et al 1997) which was supported by that subtype-specific antibodies detected M1 receptor protein in myocytes, and reverse transcriptase polymerase chain reaction detected significant M1 mRNA (Gallo et al 1993, Sharma et al 1997). However, one caveat to the report of (Gallo et al 1993)is that the effect of pirenzepine (M1 selective antagonist) in antagonizing the muscarinic stimulation of phospholipase C extrapolated to an apparent the Pkb value of approximately 9.5, which is not consistent with any known muscarinic receptor.

2.12.2. Muscarinic M2 Receptors

Muscarinic M2 receptors are found in the brain and the periphery, mainly in the heart and smooth muscle tissues. The brain muscarinic M2 receptors contribute to the centrally

mediated anti-nociception, and muscarinic M2 receptor knockout mice display disrupted agonist-induced tremor and attenuated agonist-induced hypothermia. In addition, muscarinic M2 receptors are essential for muscarinic receptors-dependent bradycardia and contribute (although only to a small extent) to agonist-induced contraction of stomach fundus, urinary bladder, and trachea (Gomeza et al 1999a, Levey 1993, Stengel et al 2000, van Koppen & Kaiser 2003).

Activation of muscarinic M2 receptors in the heart reduces force of contraction and (in non-paced tissues) a decrease in rate. These effects are caused by inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^{+} channels, respectively (Caulfield 1993). Muscarinic M2 receptors can mediate both negative and positive inotropic responses in the left atrium of the reserpinized rat, of which the positive inotropic responses are insensitive to pertussis toxin (Kenakin & Boselli 1990, Kurjak et al 1999).

2.12.3. Muscarinic M3 Receptors

Muscarinic M3 receptors occur both the brain and the periphery, mainly in glandular and smooth muscle tissue (Matsui et al 2000). The muscarinic M3 receptors play a key role in salivary secretion, pupillary constriction, and bladder detrusor contraction. Importantly, muscarinic M3 receptors are also involved in the regulation of food intake and appetite (Levey 1993, Matsui et al 2000, van Koppen & Kaiser 2003, Yamada et al 2001).

The muscarinic receptors mediating contraction of many smooth muscle preparations are defined pharmacologically as muscarinic M3 receptors (Eglen et al 1996). Muscarinic M2 receptors opposes the relaxation caused by the elevated cAMP (Eglen et al 1994, Thomas et al 1993). Muscarinic M2 receptors depolarize the muscle cells by opening of cation-selective channels in the guinea-pig ileum (Bolton & Zholos 1997).

In several studies, muscarinic M3 receptors have been shown to cause relaxation of vascular smooth muscle by the release of relaxing factors from endothelial cells (Bolton & Zholos 1997, Caulfield 1993, van Zwieten & Doods 1995). The smooth muscle muscarinic M3 receptors from different tissues may be heterogeneous. Thus, in tissues such as trachea, ileum, and urinary bladder, compounds such as zamifenacin, darifenacin, and p-F-HHSiD have been

reported to distinguish between muscarinic agonist responses (Caulfield & Birdsall 1998, Eglen et al 1996).

2.12.4. Muscarinic M4 Receptors

Like the muscarinic M1 receptors, muscarinic M4 receptors modulate central dopaminergic responses (Gomeza et al 1999b). Muscarinic M4 receptors-deficient mice show an increase in basal locomotor activity and enhancement of locomotor responses after activation of D1 dopamine receptors. Muscarinic M4 receptors, however, appear to play a negligible role in regulating peripheral smooth muscle tone (Gomeza et al 1999b, Stengel et al 2000, van Koppen & Kaiser 2003). Muscarinic M4 receptors can be detected readily in radioligand binding assays (Lazareno et al 1990). Inhibition of adenylyl cyclase activity by muscarinic agonists in rat corpus striatum probably is mediated by muscarinic M4 receptors (Caulfield 1993, Caulfield & Birdsall 1998, Olianas et al 1996).

2.12.5. Muscarinic M5 Receptors

The functionally least well-defined muscarinic receptor subtype is the muscarinic M5 receptor. Some roles have been defined in the central nervous system. Here, muscarinic M5 receptors facilitate acetylcholine-induced dopamine release in the striatum and modulate both morphine reward and withdrawal processes (Yamada et al 2001). In addition, muscarinic M5 receptors cause dilatation of cerebral blood arteries and arterioles (Basile et al 2002, van Koppen & Kaiser 2003, Yamada et al 2001).

The A2058 human melanoma cell line studies (Kohn et al 1996) and the eosinophilic leukaemia cell line (EoL-1) studies have shown functional effects of the receptor (Flynn et al 1997, Mita et al 1996, Weiner et al 1990). Even though the function muscarinic M5 receptors

is occult, the presence of the M5 protein and its mRNA in the brain and periphery have been known for more than a decade (Caulfield & Birdsall 1998).

2.13. Discussion about Muscarinic Receptors

Overall, muscarinic receptors occur widely in the body. In some organs, such as the urinary bladder, the salivary glands, the gastrointestinal tract, the eye, and the heart they play obvious and fundamental roles. In central nervous system muscarinic M1, M4 and M5 receptors occur in large numbers. Here, muscarinic M2 receptors are found presynaptically and act as autoreceptors, which are involved in the complex mechanisms like in behavioural responses by interacting with dopaminergic pathway (Clark 2012, Gamberini et al 2012, Goodman et al 2011, Rang 2007). Early studies showed that the muscarinic receptor subtypes differed in the heart and in smooth muscle tissues. In the heart muscarinic M2 receptors were found to be expressed, while muscarinic M3 receptors were found in the urinary bladder and in the gastrointestinal tract (Clark 2012, Goodman et al 2011, Rang 2007).

Lately, the physiological roles of muscarinic M1-5 receptors have been studied in knock-out mice (Dencker et al 2012). These studies have eventually shown that acetylcholine decreases the frequency by acting on muscarinic M2 receptors in the atria. Acetylcholine evokes bladder contractions by effects via the muscarinic M3 receptor and also muscarinic M2 receptors contribute by indirectly inhibiting bladder relaxatory stimuli.

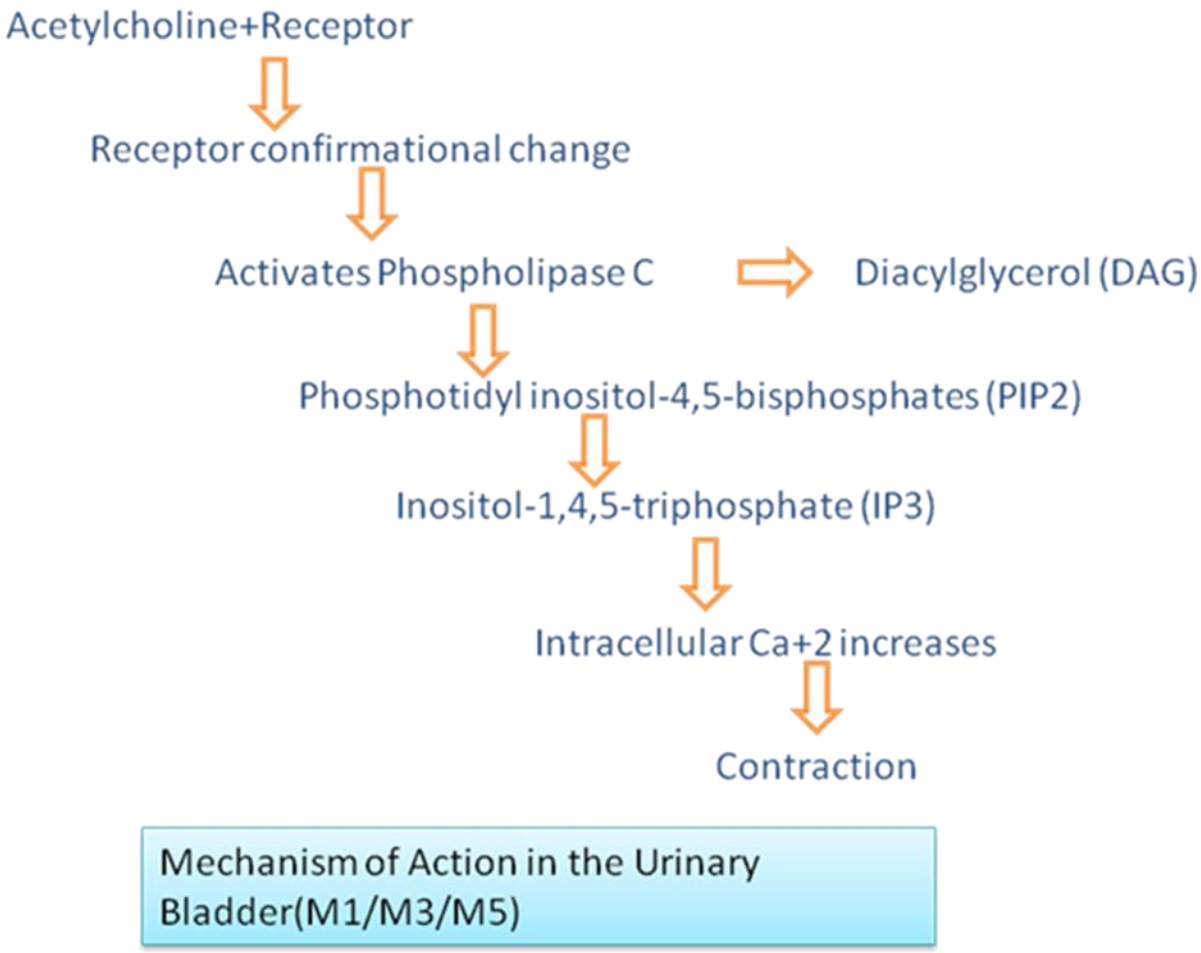


Figure 14. Mechanism of Action in Urinary Bladder

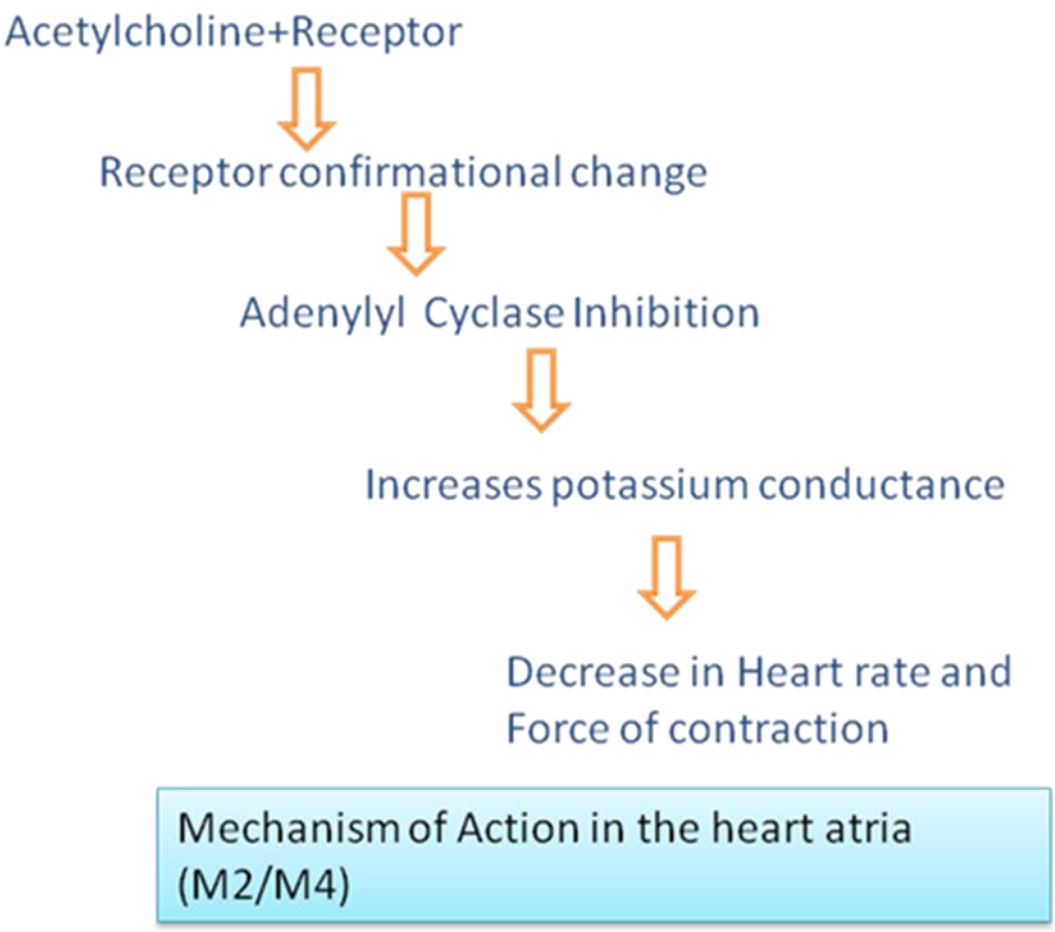


Figure 15. Mechanism of Action in Atria

2.14. Muscarinic receptors in the urinary bladder

Lower urinary tract functions, storage and voiding, are unique in that fluid has to be expelled at intervals. The autonomic nervous system and the somatic nervous system innervate the lower urinary tract (Uvelius 2001, Uvelius & Gabella 1980). The urinary bladder is divided into portions of supratrigonal, neck and trigone. The bladder wall consists of the serosa, smooth muscle, extracellular matrix (ECM), the lamina propria, and the urothelium (el-Badawi & Schenk 1966, Uvelius 2001, Uvelius & Gabella 1980).

The parasympathetic nervous system controls human detrusor contractility (Fry et al 2004) predominantly via acetylcholine acting on muscarinic receptors (Gillespie 2004a, Gillespie 2004b). The urinary bladder contains all the five muscarinic receptor subtypes. Muscarinic M2 receptors are more frequent compared to muscarinic M3 receptors at a ratio of

3:1. Muscarinic M3 receptors mediate bladder contraction *in vitro*, which is supported with functional affinity studies and M3 knockout mice studies (Abrams et al 2006, Fetscher et al 2002, Chess-Williams 2002, Chess-Williams et al 2001, Matsui et al 2000). The functional roles of detrusor muscarinic M2 receptor remain unclear. Double knockout mice studies supports that the muscarinic M2 receptor enhance contractile responses to muscarinic M3 receptor activation indirectly by inhibiting beta-adrenoceptor- (sympathetic) and purinoceptors-mediated relaxations (Giglio & Tobin 2009).

In resemblance with the gut, interstitial cells have been detected to occur in the bladder as well. *In-vitro* studies emphasised the role of interstitial cells, which are located within the suburothelial layer (Hegde & Eglen 1999, Hegde et al 1997). The interstitial cells have been suggested to have contractile and signalling properties and participate in the autonomic regulation of detrusor muscle function. The muscarinic M3 receptors present on interstitial cells affect the contractile responses. These receptors produce phasic contractions by the acetylcholine generated and released from the urothelium/suburothelium (Gillespie et al 2003). Urothelium/suburothelium also contain ATP and peptidergic neurons, which seem to play key roles in urinary bladder activity. ATP was shown by Birder et al., 2003 (Birder et al 2003) to be one such compound. In the experiments on feline urothelial cells ATP was released upon cholinergic receptor activation. Interestingly, detrusor overactivity seems to at least partly occur because of abnormal phasic activity.

Furthermore, cholinergic nerve terminals of the urinary bladder exhibit prejunctional muscarinic receptors (M1 receptors facilitate transmitter release and M2/M4 receptors inhibit transmitter release), but their functional roles are not fully understood. However, Tobin (Tobin 1995, Tobin 1998) suggested that the short-lasting facilitation serves to amplify any stimulus in order to initiate a response. Furthermore, such prejunctional receptors exhibit plasticity following spinal cord injury. In bladders of chronic spinal cord transected rats high-affinity prejunctional M3 receptors are upregulated and replace low-affinity M1 muscarinic receptors at cholinergic nerve endings (Somogyi & de Groat 1999, Somogyi et al 1996, Somogyi et al 2003). In pathological conditions of bladder denervation or spinal cord injury M2 and M4 prejunctional receptors may function in promoting urine storage (Ehlert et al 2005, Chapple 2000, LoBuglio & Neidhart 1976, Yoshida et al 2004, Zhou et al 2002).

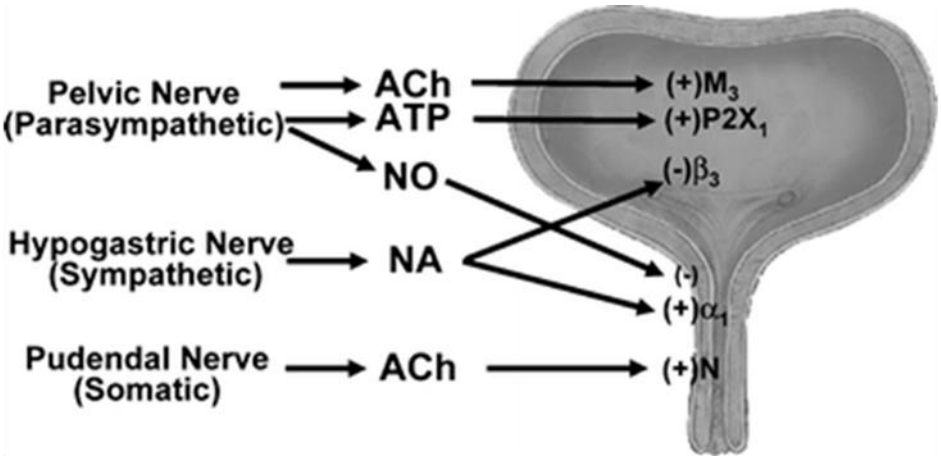


Figure 16. Innervation of lower urinary tract with muscarinic, purinergic, beta-adrenergic receptors.

The parasympathetic nerve (pelvic nerve) stimulates the bladder detrusor muscle. Acetylcholine activates muscarinic (M3) receptors and ATP activates purinergic receptors (P2X1). Nitric oxide relaxes urethral smooth muscle.

The sympathetic nerve (hypogastric nerve) stimulates urethral smooth muscle mediated by α1-adrenergic and inhibits bladder detrusor mediated by β3-adrenergic.

The somatic nerve (pudendal nerve) stimulates striated muscle of the external urethral sphincter. Nicotinic acetylcholine receptor mediates the response by Acetylcholine.

\+ indicates neural stimulation

\- indicates neural inhibition (Yoshimura et al 2008)

2.15. Muscarinic receptors in the heart

The parasympathetic nervous system participates in the regulation of the cardiovascular function, since muscarinic receptors decrease the heart rate when stimulated. However, the parasympathetic nervous system has rather limited influence on the cardiovascular system with the exception of the effects on heart rate. Here, the muscarinic stimulation changes the sinoatrial node electrical activity, which in the end leads to decrease in the cardiac output (Hulme et al 1990).

Even so, all types of muscarinic receptors are expressed throughout the cardio-vascular system, but still muscarinic M2 receptors within the heart play the most significant role in parasympathetic cardiovascular responses (Abrams et al 2006). Muscarinic M2 receptor activation can also affect the atrioventricular node (conduction of electrical impulses). In the vasculature muscarinic M3 and M5 receptor activation on endothelial cells can cause

vasorelaxation via the release of substances such as nitric oxide. In the absence of endothelium the receptors cause vasoconstriction instead (Furchgott & Zawadzki 1980, Harvey 2012).

The negative inotropic effect caused by acetylcholine acting on muscarinic M2 receptors is the result of the modulation of pacemaker activity and atrioventricular conduction (Li et al 2010). Studies on muscarinic M2 receptor knockout mice revealed the functional importance of this subtype (Gomeza et al 1999a, Lamping et al 2004). Muscarinic M1, M3 and M5 receptors are also present in the human heart but their functional roles are not unravelled (Trendelenburg et al 2003). However, in rodent cardiac tissue and human atrial myocytes muscarinic M1 receptors increase heart rate. This effect is probably indirect effects due to stimulation of catecholamine release from sympathetic neurons. Functional M3 receptors may also have some involvement in cardiac physiology as judged by knockout mice studies (Dhein et al 2001).

The role of muscarinic receptor subtypes in cardiac function (Hardouin et al 2002, Islam et al 1998) may alter in pathological conditions. This is, however, not completely unravelled and must be more thoroughly studied. In experimental congestive heart failure, muscarinic M3 receptor density increases, while the density of muscarinic M2 receptors decreases. In human coronary arteries muscarinic M2 and M3 receptor genes are expressed (Hellgren et al 2000, Ponicke et al 2003) but the functional importance of these receptors is unknown (Colecraft et al 1998, Niihashi et al 2000, Wang et al 2001, Watson et al 1996, Willmy-Matthes et al 2003).

In the mouse coronary arteries, acetylcholine induces (endothelium-dependent) dilatation when knocking out the muscarinic M2, M3 and M5 receptors (Stengel et al 2000, Stengel et al 2002). This means that yet another muscarinic receptor subtype (e.g. M1 or M4) may be involved. Further work is required to elucidate the role of other muscarinic receptor subtypes in the heart and how this may be altered in disease states.

2.16. Muscarinic receptors in the Respiratory Tract

Parasympathetic nerves controls the airways dominantly by the release of acetylcholine which activates postjunctional muscarinic receptors present on the airway smooth muscle to

cause bronchoconstriction, submucosal glands to cause mucus secretion and blood vessels to cause vasodilation (Bellingham & Ireland 2002).

The functional effect of parasympathetic stimulation in animal models of asthma is unchanged in spite of altered postjunctional muscarinic responses (Barnes 1989). This has been suggested to be caused by increasing acetylcholine concentrations due to dysfunctional inhibitory muscarinic M2 receptors at prejunctional neurons. Lack of muscarinic M2 receptors relates to asthma in humans (Fernandes et al 1992).

Muscarinic receptors have potent excitatory effects on medullary respiratory neurons and motor neurons. The parenchymal lung strip is the model of peripheral airway smooth muscle, which was introduced 15 years ago (Roffel et al 1990, Wong et al 1992). Muscarinic receptors mediate mucus hypersecretion as well as smooth muscle constriction. In the nose, the parasympathetic nervous system causes hypersecretion and vasodilation (White 1995). Muscarinic M3 receptors mediate airway smooth muscle contraction in bovine trachea, (Roffel et al 1990) guinea pig trachea, human small bronchi (Barnes 1989) and whole rat lung (Fryer & Jacoby 1998) Mixtures of muscarinic M1 and M3 receptors were found in human peripheral lung tissue (Coulson & Fryer 2003). In human lungs muscarinic M1 receptors are found on alveolar walls and the M3 subtype is present in the airway smooth muscle. In guinea pig lung muscarinic M2 receptors are also present along with muscarinic M1 and M3 receptors (Fryer & Jacoby 1998). Radioligand binding studies suggested that the M1 subtype (70-80%) are present in rabbit peripheral lung tissue (Martos et al 1987, Roffel et al 1988, Roffel et al 1993).

Anticholinergics are often used in the airway disorder treatments (Jacoby and Fryer 1998). In acute asthma anticholinergics are used to reduce the cholinergic bronchoconstriction. Organophosphates cause vagal bronchoconstriction by decreasing the function of neuronal muscarinic M2 receptors (which inhibit the release of acetylcholine) and independent of acetylcholinesterase inhibition (Lein & Fryer 2005). Muscarinic receptors may not be affected in this way by different organophosphates (OP).

2.17. Muscarinic Receptors in the Salivary Glands

The salivary glands are widely distributed through the oral mucosa. In addition to these glands, three major paired glands exists (Bloom et al 1987, Garrett 1987, Garrett et al 1998). The parotid glands, being serous, are located close to the ears, the submandibular glands, being sero-mucus, are located close to the mandible, while the mucous sublingual glands are located under the tongue. While the small accessory glands may be spontaneously active, the paired glands are completely regulated by the autonomic nervous system. By this regulation, the interplay between the parasympathetic and sympathetic nervous systems differ in comparison with many other organs. Namely, both stimulate secretion.

The parasympathetic innervation induces a perfuse secretion, poor in protein. The sympathetic stimulation causes a small secretion relatively rich of protein. The parasympathetic transmitter acetylcholine mainly activates muscarinic M3 receptors (Tobin 1995), while the sympathetic nerves release noradrenaline that acts on α- and β- adrenoceptors, while α-adrenoceptors causes fluid secretion, the β- adrenoceptors induce a very protein rich but sparse secretion (Tobin et al 2009).

Thus the muscarinic M3 receptor is the principal receptor for salivary secretion. However, muscarinic M1 receptors may also contribute (Tobin et al 2006). In the perspective of dry mouth being a common side effect to many pharmacotherapies, the heterogeneity of the muscarinic receptor has gained great interests over the years.

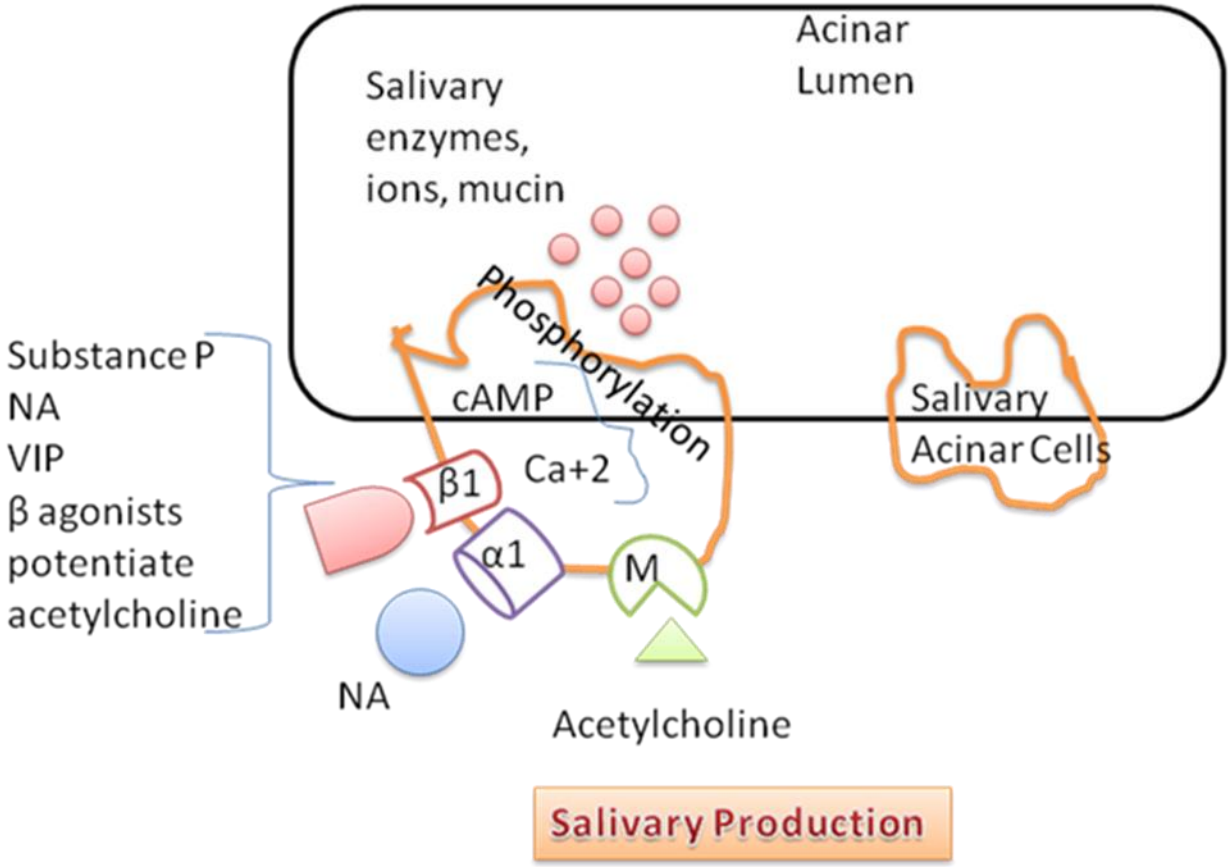


Figure 17. Production of Saliva (http://www.zuniv.net/physiology/book/chapter22.html).

2.18. Drugs

A drug is a chemical compound that affects physiological function in a cell. Drugs act on target proteins, i.e., receptors, enzymes, carriers, and ion channels. Drug must possess specificity i.e. they must bind to specific receptors only, but unfortunately no drug has complete specificity. Drugs can be administered by different routes like oral, sublingual, inhalation, injection, topical, transdermal, rectal. Drug disposition includes absorption, distribution, metabolism, excretion. Drug effects could be acute physiological responses or delayed gene manipulations.

To measure the pharmacological effect of a drug, bioassay is used. Bioassay is, the biological response measurement, helps to evaluate the concentration or potency of a substance. In drug-receptor interactions there will be two steps called binding and activation. Drugs can

be called as agonist or antagonists based on the two properties called *affinity* (i.e. binding to receptors) and *efficacy* (i.e. ability, once bound, to initiate changes that lead to effects). Efficacy is zero for antagonists (Clark 2012, Goodman et al 2011, Rang 2007).

Based on the functional effects produced by the ligands on interactions with proteins, so called receptors, ligands are characterised and classified as full agonists, partial agonists, neutral antagonists or inverse agonists. Intrinsic efficacy is defined as stimulus produced by ligand per receptor molecule (Urban et al 2007).

EC_{50} is the half of the effective maximal concentration of a drug. To determine the potency of drugs EC_{50} is used. Potency and EC_{50} are inversely related i.e. less EC_{50} more potency. The therapeutic window of a drug plays a key role in a clinical use. More the therapeutic index less are the side effects (Clark 2012, Goodman et al 2011, Rang 2007).

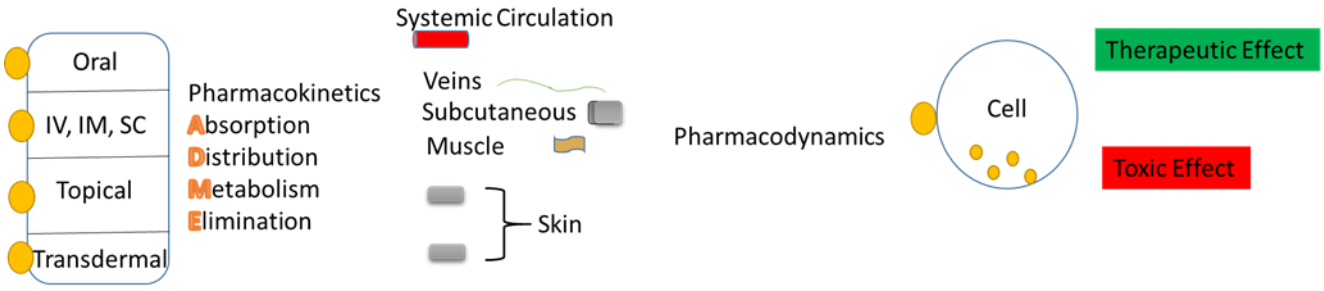


Figure 18. Drug interactions in the body

2.18.1. Agonists

Agonists stimulate a target protein which leads to cell signalling cascades on binding, whereas antagonists binds to target proteins without stimulating target proteins, but still interfering with the effect of an agonist. Some agonists provides maximal response at less occupancy which could be because of *spare receptors* which are involved in smooth muscle contraction. Agonists could be *full agonists*, having high efficacy, which produce maximum effects in the cell and the *partial agonists*, having intermediate efficacy which produce only submaximal effects within cell. *Inverse agonists* reduce the receptor activation thus reduces the efficacy (Urban et al 2007, Zhu 2005). Examples of agonists are apomorphine (dopaminergic receptor agonist) (Gamberini et al 2012), adrenaline (adrenergic receptor agonist).

In other words partial agonists are the drugs, which are cannot produce their maximum response on binding to the receptor. Example for partial agonists is xanomeline(McKinzie and Bymaster 2012). Inverse agonists reduces the action of drugs. Pimavanserin (ACP-103) which is an $5HT_{2A}$ inverse agonist and highly selective (Khilnani & Khilnani 2011).

2.18.2. Antagonists

Antagonists occupy the receptor protein but they do not exert any response. They just block the active site of the receptor not to interact with agonists. Antagonsims can be chemical, receptor block, physiological, pharmacokinetic and blockade of linkage between receptor and effector (non-competitive). Chemical antagonism occurs when chelators (dimercaprol) bind to heavy metals to reduce their toxicity. Reversible competitive antagonism and irreversible competitive antagonism constitutes receptor block antagonism. For the receptor classification reversible competitive antagonism is widely used. Pharmacokinetic antagonism involves the concentration reduction of active drug (warfarin) at the site of action by the antagonists (phenobarbital). Physiological antagonism is, the physiological effects produced by two drugs are get neutralised by each other. Examples for antagonists are exogenous nitric oxide (NO) donor l-arginine Ca ($^{2+}$) channel antagonist nifedipine (30nM) (Dai et al 2012). Cholinesterase inhibitor (neostigmine), a muscarinic receptor antagonist (atropine), and a nicotinic acetylcholine receptor antagonist (hexamethonium)(Tangsucharit et al 2012). These antagonists could be reversible and irreversible.

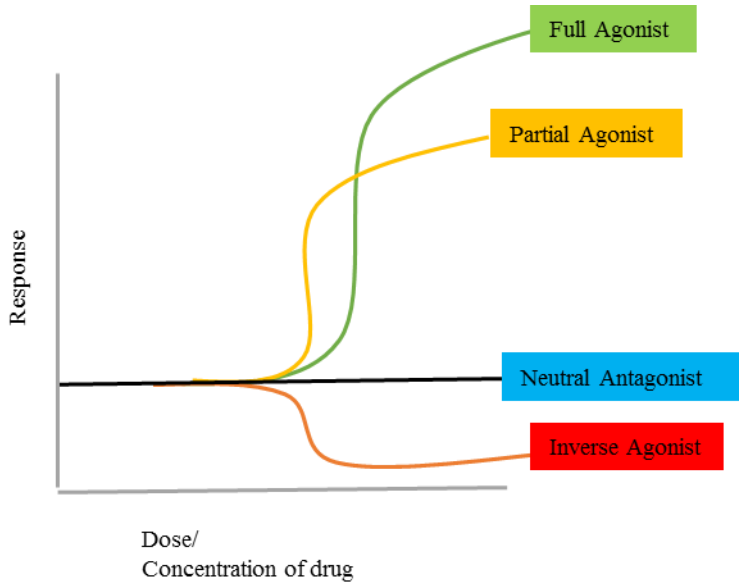


Figure 19. Typical dose response curves of drugs

2.19. Cholinergic Drugs

2.1.1. Muscarinic Agonists

Pharmaceuticals targeting the muscarinic receptors may be employed in order to treat many disorders. Based on what has been discussed so far in this introductory review, parasympathetic regulation affects many functions and acetylcholine acting on muscarinic receptors plays an essential roles.

All muscarinic agonists are similar to acetylcholine in structure. Muscarinic agonists contains the positively charged quaternary ammonium group and partially negatively charged ester group. Most of the agonists binds the receptors at the orthosteric site (Gregory et al 2007). Muscarinic agonists, decreases the heart rate, causes the vasodilation (Harvey 2012), smooth muscle contraction, lacrimation, sweating, salivation, bronchial secretion, contraction of ciliary muscle (Clark 2012, Goodman et al 2011, Rang 2007). Normally muscarinic (M1) agonist lack specificity, low bioavailability, low therapeutic window (Fisher et al 2002). So muscarinic agonist's clinical uses are limited. To treat glaucoma pilocarpine, muscarinic agonist, is used clinically (Clark 2012, Fisher et al 2002, Goodman et al 2011, Rang 2007). Allosteric

muscarinic agonists could be helpful to treat several neurological disorders (Ehlert et al 1994). Understanding the allosteric modulation of muscarinic receptors by the agonists is very much needed to provide novel therapies for dreadful diseases.

2.1.2. Muscarinic Antagonists

Muscarinic antagonists contain the quaternary/tertiary ammonium group with a big aromatic group and an ester group. They are lipid soluble. Muscarinic antagonists bind to the orthosteric site of the receptors to block the active site of receptor. Their effects on heart causes tachycardia, stops the secretions like saliva, tears, dilation of pupil, bronchial dilation, on central nervous system it causes excitation. The muscarinic antagonists clinically used to treat peptic ulcer, asthma, bronchitis, urinary incontinence (Clark 2012, Goodman et al 2011, Rang 2007).

Classical muscarinic antagonists include atropine, tiotropium, pirenzipine, darifenacine, ipratropium bromide, scopolamine (Baysinger et al 2012, Clark 2012, Goodman et al 2011, Martos et al 1987, Rang 2007). 'Neural' muscarinic M1 receptors produce slow excitation of ganglia. Muscarinic M1 receptors have high affinity for pirenzepine and also for biperiden (Klinkenberg et al 2013). Gallamine is suggested to decrease cardiac rate and force of contraction (mainly of atria) because it blocks muscarinic M2 receptors (Dai et al 2012). Selective muscarinic antagonists include tripitramine (Gamberini et al 2012) and methoctramine (Angeli et al 1995, Giglio et al 2007).

Novel cardio-selective muscarinic antagonists are AF-DX 116 (Martos et al 1987) and 4-DAMP (4-diphenlacetoxy-N-methylpiperidine methiodide) which is selective to muscarinic M1, M3 and M5 receptors (Scarpero & Dmochowski 2003). Darifenacin and hexahydrosiladifenidol are also muscarinic M3 receptor selective antagonists (Kurjak et al 1999). In the treatment of over active bladder, clinically, muscarinic antagonists are used (Harvey 2012). Tropicamide (M (4) antagonists) is another muscarinic antagonist often used in studies for muscarinic receptor subtypes (Pelegrina et al 2013).

2.1.2.1. Methoctramine

Methoctramine (N, N'-bis [6-[[(2-methoxybenzyl)-amino] hexyl]-1, 8-octane] diamine) is a polymethylene tetraamine.

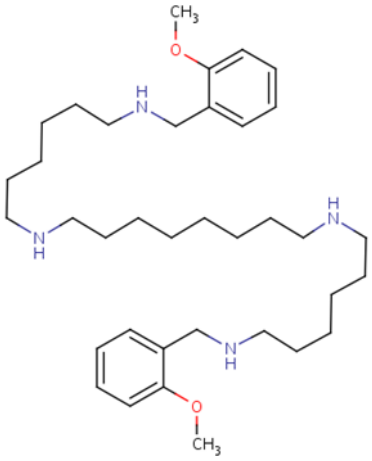


Figure 20. Structure of Methoctramine
(http://www.ebi.ac.uk/chebi/chebiOntology.do?treeView=true&chebiId=CHEBI:73339#graphView)

Methoctramine is a cardioselective M2 selective competitive antagonist of muscarinic receptors (Wess et al 1988). It shows allosteric property at high concentrations. Methoctramine shows selectivity to M(2) receptors because of interactions with both the orthosteric and the allosteric binding sites located between the second and third extracellular loops (Jakubík & El-Fakahany 2010). However, methoctramine may also acts as an antagonist on nicotinic receptors in the airways (Watson et al 1992). This is major tool for the functional studies of muscarinic receptors subtype M2 because of its relative specificity. It may, however, show some cytotoxicity effects at high concentrations (Zini et al 2009). M2 receptor agonists and acetylcholinesterase inhibitors may be the potential targets to reduce nociceptive transmission from orofacial tissues (Jeong et al 2013). Methoctramine is a potent muscarinic M2 receptor antagonists, but unable to differentiate the cholinoceptor (muscarinic and nicotinic) subtypes in airways (Watson et al 1992).

2.1.2.2. Atropine

Atropine is a tropane alkaloid extracted from Atropa belladonna. Atropine is a competitive antagonist of the muscarinic receptors and blocks for instance the acetylcholine

vascular relaxation selectively (Choi et al 1999, Kumari et al 2007). Anticholinergics have potential psychoactive properties. For instance, atropine affects the synthesis of serotonin in the serotonergic pathways (Kumari et al 2007).

Atropine is an important tool to understand the neurobiology of the cholinergic system, because of its anticholinergic property at postganglionic parasympathetic muscarinic sites. For the physiological studies, behavioural studies, neurological studies muscarinic receptors are the important tools and to characterise muscarinic receptors atropine and other anticholinergics are very important drugs for research studies.

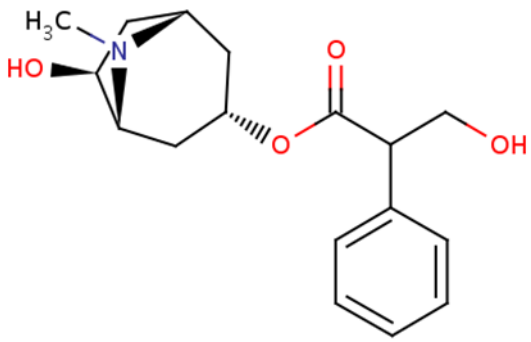


Figure 21. Structure of Atropine
(http://www.ebi.ac.uk/chebi/chebiOntology.do?treeView=true&chebiId=CHEBI:73339#graphView)

Atropine affects the heart rate. In very low doses it causes bradycardia. Atropine (2-3mg/kg) blocks the acetylcholine mediated vagal input to the sino atrial node. Muscarinic receptors occur on the ciliary muscles of the lens and sphincter muscle of iris. On stimulation they cause pupillary constriction and accommodation for clear near vision (Penetar 1990).

2.20. Reactivators

Oxime reactivators are the compounds, which reactivate the inhibited acetylcholine esterase enzyme (EC 3.1.1.7). The reactivators are used in the treatment of organophosphate poisonings (OPPs) and carbamates poisonings. The organophosphate compounds are highly toxic compounds and can be nerve agents or pesticides. The organophosphates act on the serine hydroxyl group of the acetylcholinesterase enzyme. By this they inhibit the enzyme by phosphorylation or phosphonylation (Jun et al 2011, Soukup et al 2010a). Oximes include

obidoxime, pralidoxime, tremidoxime, methoxime and HI-6 (Soukup et al 2013, Soukup et al 2010a). The allosteric antagonistic property of obidoxime may be of importance in investigations for other actions of the oximes. Other reactivators which are of interest in the drug development are HI-6 (bisquaternary oxime), 1-(2-hydroxyamino-methylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dimethansulfonate. These may be antidotes for VX, soman, sarin organophosphate poisonings (Soukup et al 2008). Many reactivators seem to have an anti-muscarinic effect at high concentrations and to cause acetylcholinesterase inhibition (contractile response) at lower concentrations (Soukup et al 2011b, Soukup et al 2008). However, photoisomerism is exhibited by HI-6 (Bogan et al 2012). Other reactivators are tremidoxime (bisquaternary oxime), an antidote for tabun and paraoxan used in organophosphate poisonings (Soukup et al 2010a).

2.20.1. Obidoxime

Obidoxime (1, 3-bis (4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride), is a well-known bis-pyridinium reactivator. It is the preferred antidote of organophosphorus poisoning caused by pesticides and tabun, but a poor reactivator of soman- and cyclosarin inhibited acetylcholinesterase. Notably and which is of particular interest in the current experiments is that obidoxime is an allosteric antagonist of the muscarinic M2 receptor (Ellis & Seidenberg 1992, Grossmuller et al 2006).

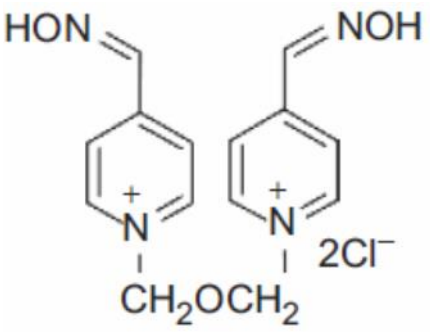


Figure 22: Structure of Obidoxime

2.21. Cholinesterase Inhibitors/ Anticholinesterases

Cholinesterase inhibitor inhibits the acetylcholinesterase, which thus cause an increase the availability of acetylcholine and its action in the synaptic cleft. Cholinesterase inhibitors are divided into two classes, which includes organophosphorus compounds and carbamates. Organophosphorus compounds seem to be more toxic and have a longer duration of action. Carbamates are less toxic and have a rather short duration of action. The structural and functional differences are depicted in the following figure 22. Rivastigmine is one of the cholinesterase inhibitor used to treat neurodegenerative disorders (Klinkenberg et al 2013).

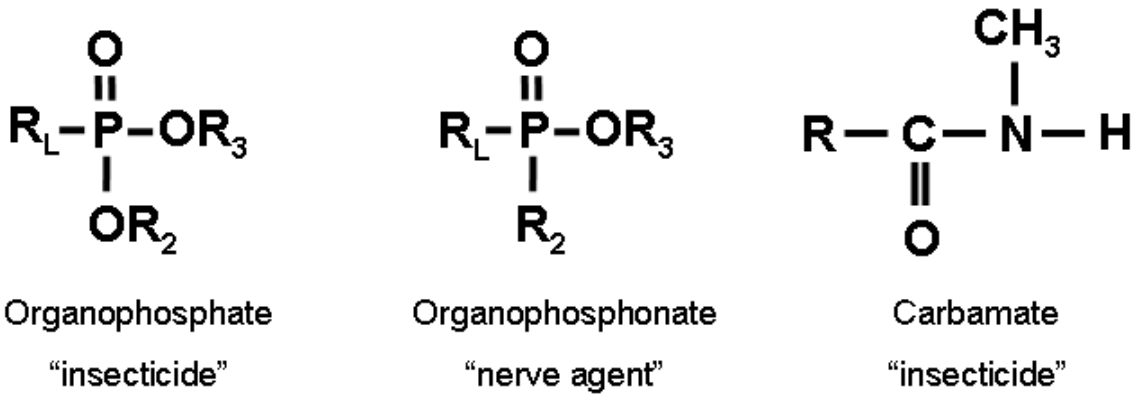


Figure 23. Ogranophospahtes having the same basic structure but the functional groups attached to the P are changed. Carbamates structure have different basic structure. (http://www.atsdr.cdc.gov/csem/csem.asp?csem=11&po=5).

When organophosphates (insecticides) act on acetylcholinesterase enzyme they act on active site, which is partially electronegative.

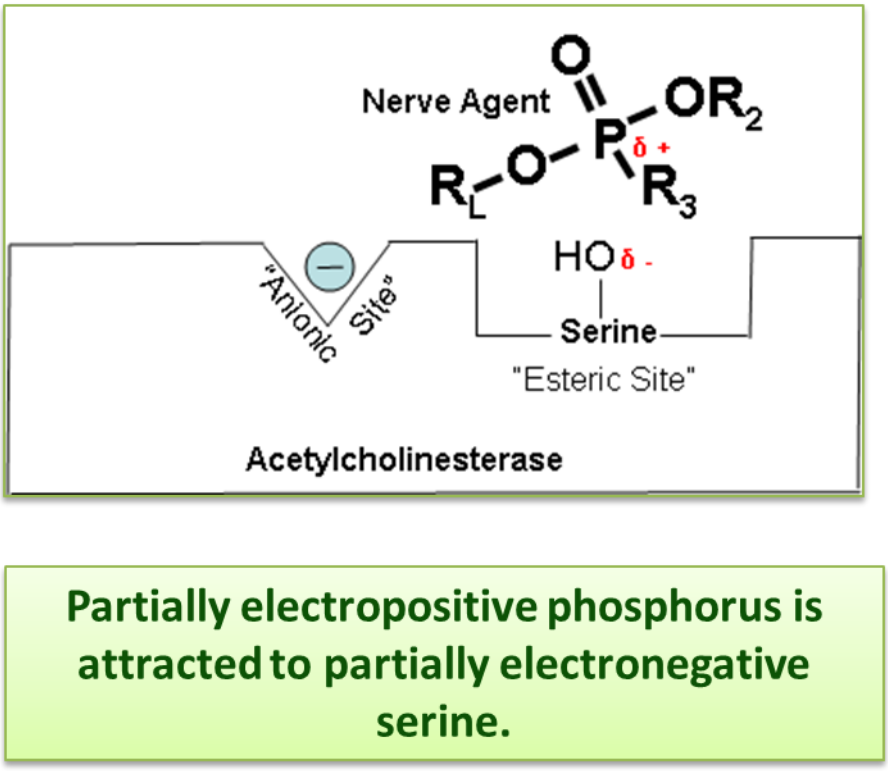


Figure 24. Partially electropositive phosphorous present in OPPs is attracted towards serine which is partially electronegative.

During transition, the functional group $R_{L,}$ which is attached to the electropositive phosphorous, will be broken down. The partially electropositive phosphorous and partially electronegative oxygen form the complex, which prevents the acetylcholinesterase to bind with acetylcholine. Thus organophosphate poisonings block the serine functional site in the enzyme. This is shown in the figure 25 (a) and (b).

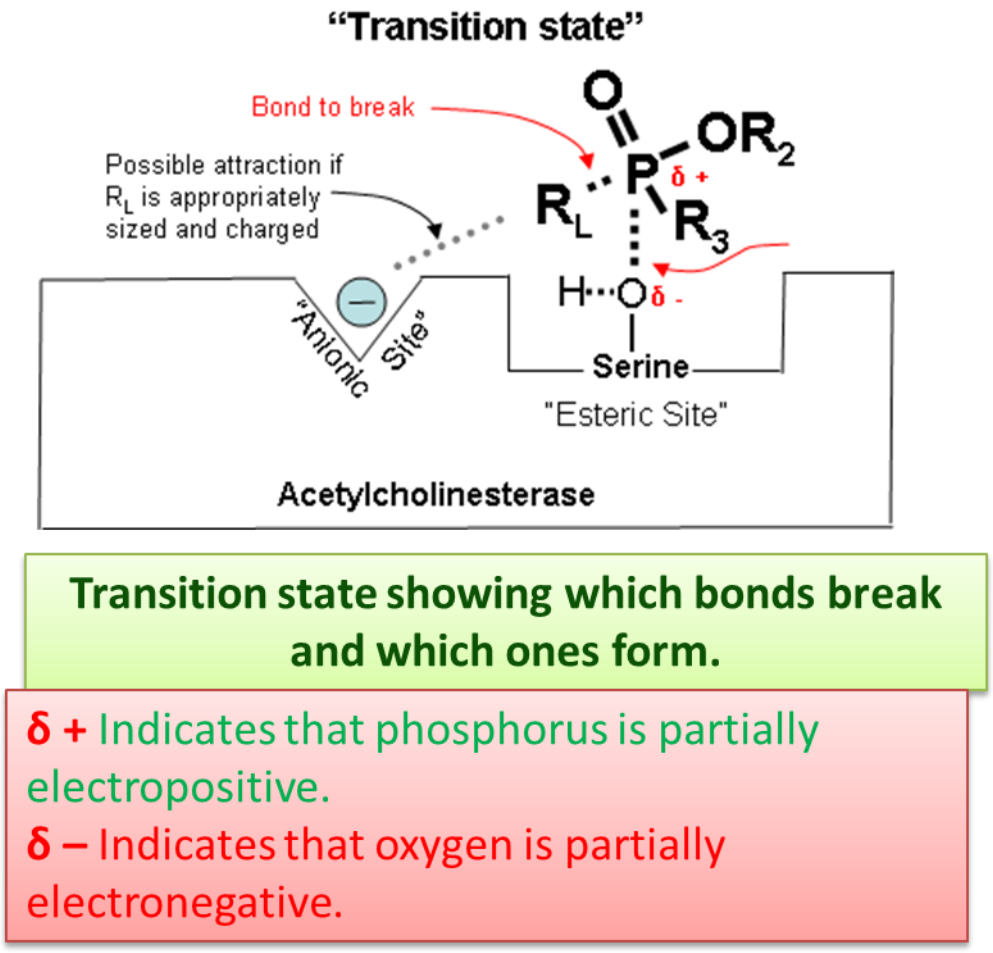


Figure 25(a) Transition state showing the bond breakage and bond formation. Electropositive phosphorous forms the bond with electronegative oxygen.

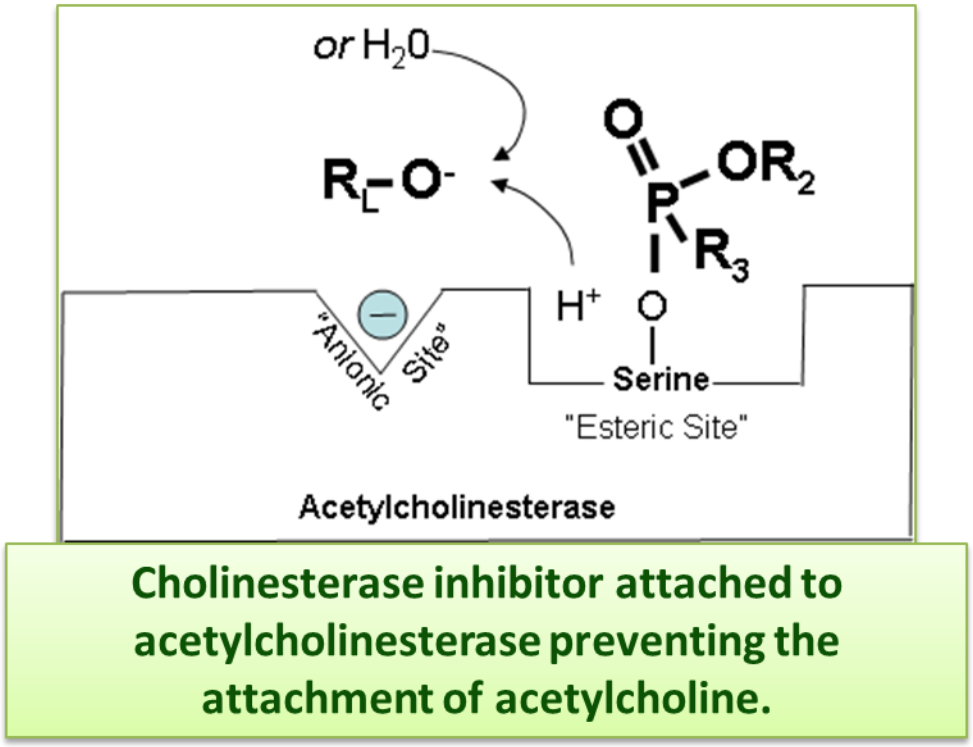


Figure 25(b)

Figure 25. (b) The bond formation between P and O forms the complex which prevents the acetylcholine attachment to the enzyme cholinesterase. (http://www.atsdr.cdc.gov/csem/csem.asp?csem=11&po=5**).**

2.21.1. Physostigmine

Physostigmine (Eserine) is an alkaloid present in physostigma venenosum (calabar bean- fabaceous plant) (Percy L. Julian & Pikl 1935). The structure of physostigmine is 1,2,2,3a,8a,-hexahydro-1,3a,8-trimethyl-pyrrolo[2,3-b]indo-5-ol-methylcarbamate. It is a carbamate inhibitor of acetylcholinesterase (Traub et al 2002).

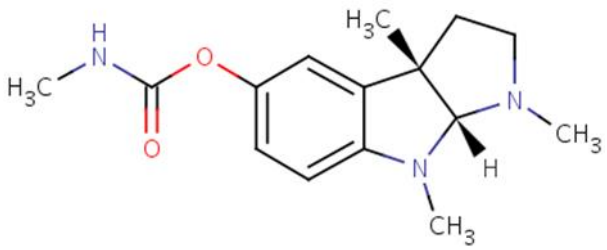


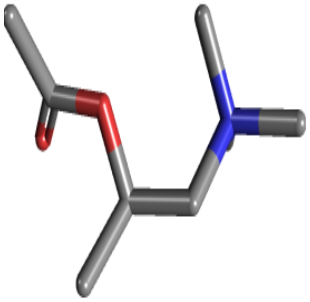
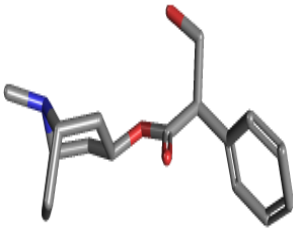
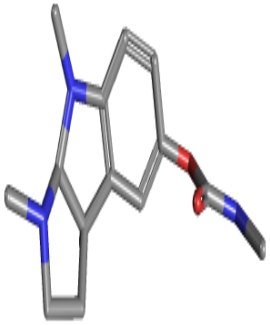
Figure 26. Structure of Physostigmine
(http://www.ebi.ac.uk/chebi/chebiOntology.do?treeView=true&chebiId=CHEBI:73339#graphView)

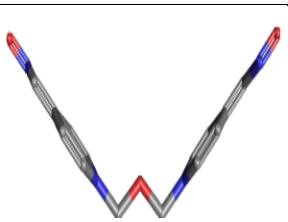
Physostigmine salicylate inhibits the acetylcholinesterase reversibly and increase the acetylcholine concentration at the synapse and stimulates the cholinoceptors; i.e., both muscarinic and nicotinic(Scheindlin 2010). Physostigmine may directly acts on acetylcholine receptor complexes, as well as interact with the acetylcholine gated cation channels. Physostigmine has short duration of action. Physostigmine is non-ionic, tertiary amine which is a lipid soluble compound. Thus physostigmine easily cross the blood brain barrier and may thus act on central nervous system. This property makes to play a key role in the treatment of alzheimer's disease. Physostigmine is clinically used to treat glaucoma, myasthenia gravis etc. Half-life of physostigmine in rat is 16min, in dog 23min where as in man it is 30min. It is metabolised in liver and the bioavailability is very low<2%. It has narrow therapeutic window (Mach et al 2004, Meshulam et al 2001, Somani & Dube 1989). As a pseudosubstrate or competitor physostigmine interacts with acetylcholinesterase, thus forms the stable intermediate drug-enzyme complex which prevents endogenous acetylcholine from reacting with the acetylcholinesterase enzyme and thus retain the local concentration of acetylcholine at the synapse (Triggle. et al 1998). In the body plasma esterases metabolise the physostigmine (V Priya Nair & Hunter. 2004). Anticholinesterases may provide a platform to develop the novel drug therapies to treat several diseases (Greig et al 2013).

2.22. Clinical use of cholinergic drugs

Anticholinergic drugs are often employed in the treatment of chronic obstructive pulmonary diseases, counteracting the smooth muscle contraction, decrease of mucus secretion and bronchial vasodilatation (Barnes 1998, Gross 1991), to treat bradycardia, to treat ulcer, urinary incontinence, asthma, bronchitis. Cholinergic drugs (agonists) have limited use especially in treating glaucoma. Cholinergic drugs having less specificity towards receptor subtypes, having adverse side effects. If we can overcome these properties then the cholinergic drugs will be the first line drugs to treat many neurodegenerative disorders, organophosphate poisoning, cardiovascular disorders, overactive bladder, chronic obstructive pulmonary disorder etc. Some of the cholinergic drugs which are interesting in research to understand the cholinergic interactions are tabulated below.

Table 2. Examples of Cholinergic drugs which are used in this thesis

Compound	Brand name	Structure	Clinical uses	Pharmacology
Methacholine	Provocholine		Methacholine Challenge Test (PMID: 6370167)	More selective to muscarinic receptors than nicotinic receptors. Cannot cross BBB. Resistance to AchE
Atropine	Atropine sulphate		As cycloplegic, To treat bradycardia, inhibit secretions from salivary glands and mucus glands. Treatment for Organo Phosphate Poisonings.	Anticholinergic drug. Used to counteract the vagus nerve stimuli on heart.
Physostigmine or Eserine	Physostigmine Salicylate (ANTILIRIUM)		Used clinically for their potentiation of cholinergic inputs to the gastrointestinal tract and urinary bladder, the eye, and skeletal	Inhibit the choline esterase enzyme

			muscles; also used for their effects on the heart and the central nervous system.	
Obidoxime	Toxigonin		Used clinically for Organo Phosphate Poisonings.	Choline esterase reactivator- Antidote. Weak inhibitor of esterase.

3. Aims

The current thesis aims are to provide a better understanding of the mechanisms by which esterase reactivators act in view of there sometimes obscure toxicity. A better understanding of the mechanisms of action, particularly with respect to the cholinergic transmission, new entities with the fewer side effects and better potency can probably be developed. Thus the specific aims were:

- To determine the functional significance of muscarinic M2 receptors in the state of acetylcholinesterase inhibition, elucidating muscarinic M2 and M3 receptor interaction.
- To study the functionally characterized cholinergic effects of obidoxime regarding muscarinic M2 and M3 receptors.
- To functionally compare obidoxime with a classical esterase inhibitor (Physostigmine).
- To further investigate the subtypes and functional role of muscarinic receptors.

4. Materials and Experimental Methods

The Swedish ethics committee at the University of Gothenburg approved the studies in which male rats (250-350g) of the Sprague-Dawley strain were used. The rats were anaesthetized with pentobarbitone (45 mg/kg i.p.®), for *in vivo* studies. For *in vitro* experiments the rats were gassed and killed with the carbon dioxide. The atria and urinary bladders were then removed from the sacrificed rats. They were used for functional examinations *in vitro* by using organ baths.

4.1. Materials

The following substances were used in the study:

Adrenaline (Sigma-Aldrich, St Louis, MO, US)

Acetyl-β-methylcholine chloride (methacholine) (Sigma-Aldrich, St Louis, MO, US)

1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride (obidoxime), (Sigma-Aldrich, St Louis, MO, US)

Physostigmine (Sigma-Aldrich, St Louis, MO, US).

Obidoxime (Toxogonine; 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride), synthesized at the Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic,

methacholine: acetyl-β-methylcholine chloride

Pentobarbitone (Apoteket AB, Sweden).

Ketalar (Apoteket AB, Sweden).

Acetylcholine chloride from Merck (Darmstadt, Germany),

Sodium Chloride (NaCl)

Potassium Chloride (KCl)

Calcium Chloride ($CaCl_2$)

Potassium dihydrogen Phosphate (KH_2PO_4)

Magnesium Sulphate ($MgSO_4$)

Sodium Bicarbonate ($NaHCO_3$) and

Glucose all other chemicals were from Sigma Aldrich (St. Louis, MO, USA).

4.2. Experimental methods

4.2.1. *In vivo* physiological and functional experiments

In vivo measurement

Male rats (220 g) were anesthetized with pentobarbitone (45 mg/kg i.p.) followed by supplementary doses injected intravenously as required during the experiments. For maintaining a free airway, a cannula was placed in the trachea of the rat after tracheotomy.

Body temperature was maintained at ~ 38°C by means of a thermostatically controlled blanket connected to a thermistor inserted into the rectum. The blood pressure and heart rate were monitored continuously via a catheter placed into the femoral artery. A cannula placed in the femoral vein was used for all drug administrations. The right vagal nerve was exposed in the neck and a bipolar electrode placed under the nerve.

Sympathetic nerve fibres running in close contact with the vagal nerve were identified and cut. By this procedure the effect of electrical stimulation of the nerve was changed from an increase to a decrease of the heart frequency. To prevent afferent activation, the vagal nerve was cut coronally to the point of stimulation.

The protocol included administration of successively larger doses of obidoxime (0.01–100 mg/kg IV) during an on-going electrical stimulation of the vagal nerve (10 Hz, 8 V square-

wave; 2 ms pulse-width). Some experiments were made in which atropine (1–1000 μg/kg IV) substituted obidoxime. The heart frequency was calculated within 1 min as the average of 4–6 peaks, just after getting a stable reading.

4.2.2. *In vitro* functional experiments

The organ bath experiments *(In vitro measurement of muscarinic receptor inhibition)*

Whole atria and urinary bladder strips were prepared from the part of the collected heart and urinary bladder for functional studies. The atrial and detrusor strip was fastened between two steel rods of which one was adjustable while the other was fixed. The former was connected to an isometric force transducer (Linton). The strips were immersed in 25ml organ bath containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.18, $MgSO_4$ 1.16, $NaHCO_3$25 and glucose 5.5 in deionized water; and which was gassed with 5% CO_2 in O_2 at 37°. All drugs were administrated to the organ bath in a volume of 125μl.

The atria were exposed to a low frequency (0.1 Hz) stimulation aiming to provoke a spontaneous activity. For the atrial preparation, the viability assessment was carried out according to the occurrence of spontaneous activity or not. In the experimental protocol, electrical field stimulation of the atria was carried out at 0.1 Hz at supramaximal voltage (50 V), delivered as square wave pulses with duration of 0.8 ms (stimulator: STM100C; Linton, Welwyn Garden City, UK). Data were recorded using an MP100WSW data acquisition system and ACQUIRE software (Biopac, Goleta, CA, USA). In order to enhance the possibility for cholinergic effects on the atrial frequency, adrenaline (0.1 mmol/L) was added initially. In pilot experiments, the methacholine effect on the atrial frequency was found to be small and inconsistent. Thereafter, the experimental protocols were more or less the same according to bladders and atria with the exception of the adrenaline presence in the atrial experiments only.

The bladder strips were repeatedly stretched to a tension of about 5-10mN and left to equilibrate for 45 min. This resulted in gradual relaxation that eventually reached a stable tension of 6-8mN. To the bladders, a high K+ solution (containing 124 mmol/L K^+ obtained by exchanging Na^+ for equimolar amounts of K^+) was administered at the beginning of each experiment to induce a reference contraction and in order to assess the viability of each

individual preparation. Data were recorded using a MP100WSW data acquisition system and Acquire software (Biopac).

Two principal types of experimental protocols were used. In one type, the agonist (methacholine) was administered cumulatively before and after the addition of acetylcholinesterase inhibitor/reactivator (physostigmine/obidoxime) and/or 'muscarinic M2 receptor-selective' antagonist (methoctramine). Methacholine was chosen because of its resistance towards acetylcholine esterase in order to minimize the direct effects of the esterase inhibition on the agonist-evoked response. In another type of experimental protocol, a standard concentration was selected (larger than EC50 and lower than Emax). This concentration was repeatedly administered in the absence and presence of atropine, physostigmine and obidoxime.

4.3. Statistical analyses

All data values are expressed as mean±SEM. Statistical significance was determined by one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparison test or by the Dunnet´s multiple-comparison test. All statistical analyses were performed on raw data, even when the graphs are presented in percentage. P-values less than 0.05 were regarded as statistically significant. Graphs were generated by using the GraphPad Prism software (GraphPad Software, Inc., San Diego, USA).

5. Results

5.1. *In vivo* effects on heart frequency by different doses of obidoxime

In the anesthetized rats, the basal heart frequency amounted to 364 ± 34 bpm (n = 8). At the onset of electrical stimulation of the vagal nerve (10 Hz), the heart frequency was instantaneously reduced to 277 ± 34 bpm (−25 ± 6%; $p < 0.01$; n = 8). In the presence of 0.01 mg/kg of obidoxime, the frequency was reduced in comparison with the basal frequency (−18 ± 8%; $p < 0.05$; n = 8), but no reduction in frequency occurred in the presence of obidoxime at 0.1 mg/kg (−13 ± 9%; n = 8). At the higher doses, significant reductions once again appeared. At 1 and 10 mg/kg IV, the frequency was reduced by −25 ± 9% ($p < 0.01$; n = 8) and −23 ± 5% ($p < 0.05$; n = 8), respectively. At the stop of stimulation, the frequency returned to the basal level (367 ± 33 bpm) (Figure 27).

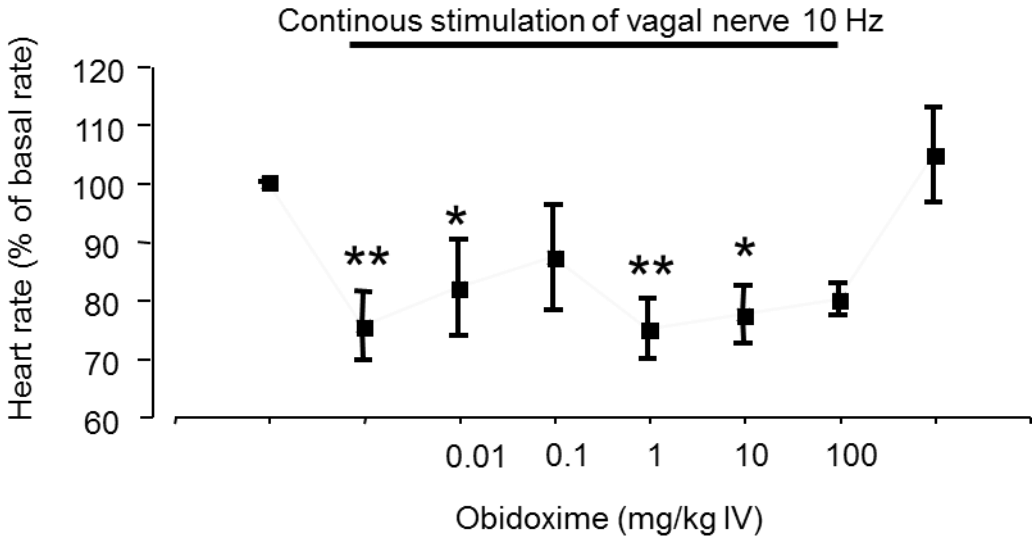


Figure 27. The effect of obidoxime on BPM in the anesthetized rat. SS stands for Stop Stimulation, i.e. BMP at the end of experiment—with all doses of obidoxime without vagal stimulation. Horizontal bar indicates the vagal stimulation. All values are expressed as mean ± SEM, stars indicate the statistical significance-diversity from the basal response: * $p < 0.05$; ** $p < 0.01$. The reduction of heart frequency in response to the on-going electrical stimulation was dose-dependently inhibited by the administration of obidoxime (0.01 and 0.1 mg/kg IV).

In the experiments (n = 3), examining the effects of atropine (Figure 28), the vagal stimulation reduced the frequency from 555 ± 8 to 515 ± 8 bpm ($p < 0.05$). At 100 μg/kg of atropine, the frequency was still significantly reduced (−7 ± 2%; $p < 0.05$) by the vagal stimulation, while atropine at larger doses (1–100 mg/kg IV) abolished the vagus-induced bradycardia.

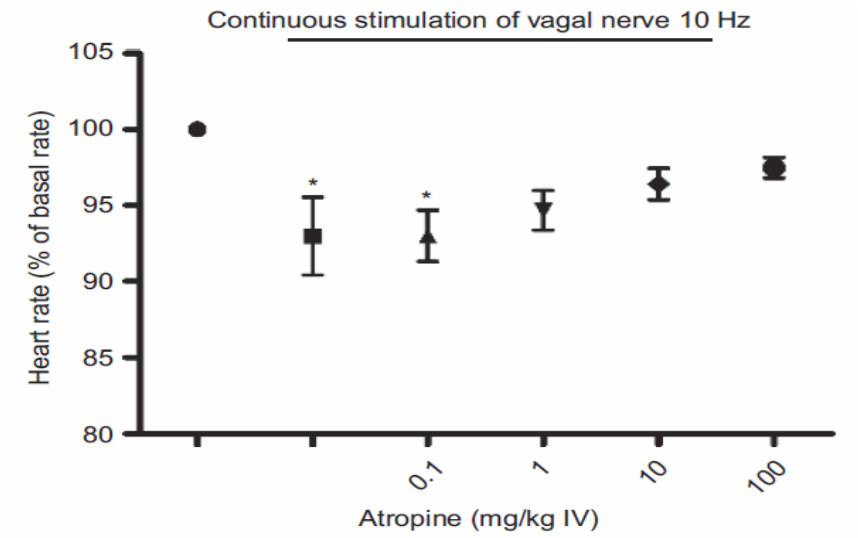


Figure 28. The effect of atropine on BPM in the anesthetized rat. Horizontal bar indicates the vagal stimulation. All values are expressed as mean ± SEM, stars indicate the statistical significance-diversity from the basal response: * $p < 0.05$.

Thus, in the experiments within the anesthetized rat, electrical stimulation of the vagal nerve activates cholinergic neurons and the effect on muscarinic M2 receptors may be blocked by atropine. Also, obidoxime showed a certain antimuscarinic effect, but only at some stimulation frequencies. At other, the vagal inhibitory effect on the heart rate seemed to be enhanced. All in all, an acetylcholinesterase reactivator shows dual effects. In a concentration-dependant manner it may either act as a weak acetylcholinesterase inhibitor or as a muscarinic receptor antagonist.

5.2. *In vitro* effects by different doses of obidoxime on atrial frequency and urinary bladder contractions I

The spontaneous (supported by electrical stimulation 0.1 Hz), basal frequency of isolated atrial preparations amounted to 551 ± 46 bpm (n = 5). After administration of adrenaline (0.1 mM), the frequency increased by 35 ± 7%. Additional administration of methacholine (0.1 mM) decreased the frequency by 31 ± 2% (to 503 ± 21 bpm; $p < 0.01$; n = 5). When obidoxime was present as well, it statistically abolished the methacholine evoked frequency reduction, except at 1 μM of obidoxime (−25 ± 3%; $p < 0.05$; n = 5) (Figure 29). In a control experiment, where only obidoxime was administered, no effect was observed on the BPM of the isolated atria.

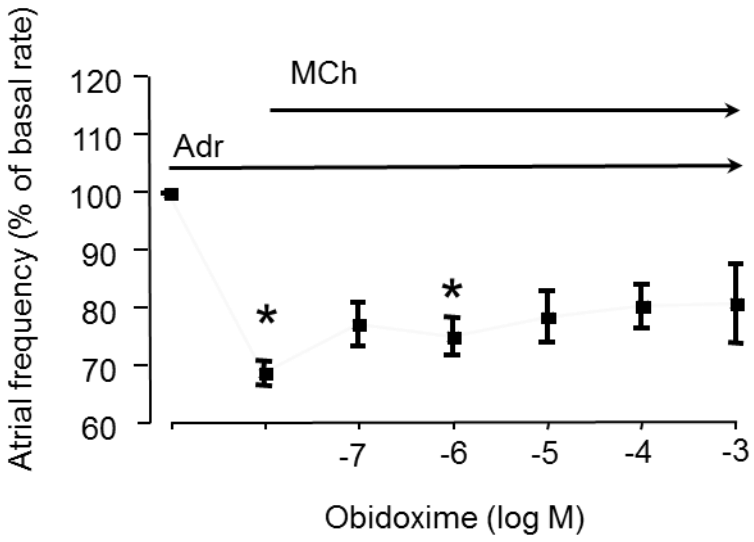


Figure 29. The effect of obidoxime on BPM in the isolated atria. Adr stands for Adrenaline (10^{-4}M); MCh for Methacholine (10^{-4}M). Horizontal arrow indicates the administration and presence of drugs. All values are expressed as mean ± SEM, stars indicate the statistical significance-diversity from the basal response: * $p < 0.05$.

In the experiments examining atropine (Figure 30), methacholine reduced the frequency of the isolated atrial preparations by 10 ± 3% ($p < 0.05$; n = 5). In the presence of any concentration of atropine (10^{-7}–10^{-3} M), methacholine caused no significant decrease. Anyhow the atropine effect seemed to concentration dependently inhibit the methacholine-evoked reductions. In the presence of atropine 10^{-7} M, the reduction was −7 ± 2% and at atropine 10^{-5} M it was −0.5 ± 4%.

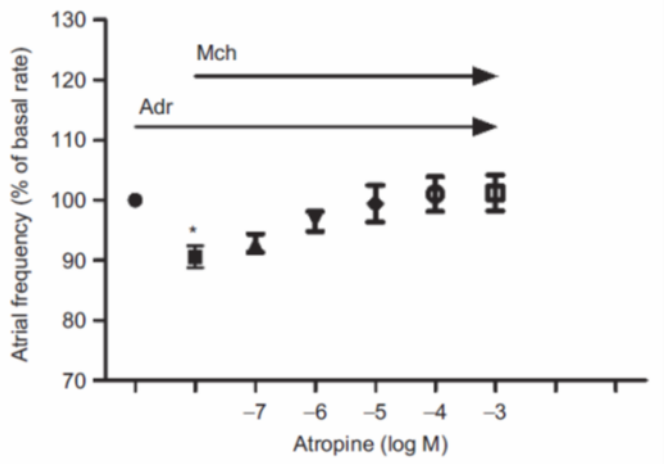


Figure 30. The effect of atropine on BPM in the isolated atria. Adr stands for Adrenaline (10^{-4}M); MCh for Methacholine (10^{-4}M). Horizontal arrow indicates the administration and presence of drugs. All values are expressed as mean ± SEM, star indicates the statistical significance-diversity from the basal response: * $p < 0.05$.

According to the generated IC_{50} from the non-lin regression fit, atropine was more potent and more efficacious than obidoxime in inhibiting methacholine-evoked frequency reduction in the atrial preparations (Table 2). The IC_{50} for atropine was 5.2×10^{-9} M and the corresponding IC_{50} for obidoxime was 8.68×10^{-2} M in the bladder preparations (Table 3).

In the in vitro atrial experiments, no or only minor influences from the autonomic nervous systems seemed to occur. Because of this adrenaline had to be administered in order to reveal any cholinergic effect; e.g. by methacholine. Also, the provoked (0.1 Hz) spontaneous beats may have made the preparations less sensitive to exogenous stimulation.

Table 3. IC_{50} values for atropine and obidoxime in the isolated atria and urinary bladder.

	Atropine	n	Obidoxime	n
Atria (M)	6.91×10^{-4}	5	2.17×10^{-2}	5
Urinary bladder (M)	5.2×10^{-9}	6	8.68×10^{-2}	6

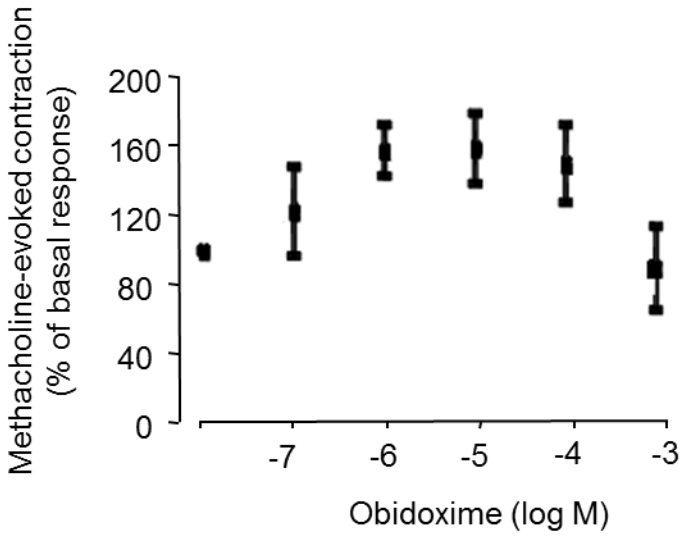


Figure 31. The effect of obidoxime on methacholine (5×10^{-6} M) evoked contraction of the bladder. All values are expressed as mean ± SEM.

In bladder preparations (Fig 31) the contraction to methacholine 5×10^{-6} M amounted to 1.43 ± 0.61 mN (n = 6). In the presence of a large concentration of obidoxime (1 mM), the methacholine-evoked contraction amounted to 89.9 ± 24% (n = 3) of basal response. Although obidoxime at lower concentrations tended to increase the contractions, if anything, it had no significant effect.

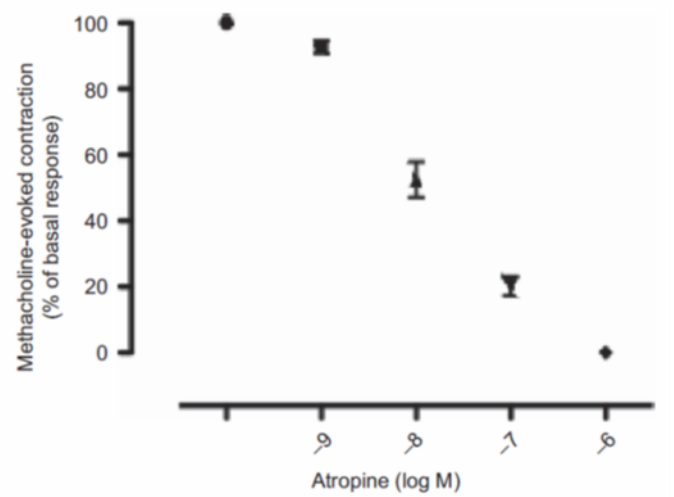


Figure 32. The effect of atropine on methacholine (5×10^{-6} M) evoked contraction of the bladder. All values are expressed as mean ± SEM. No stars indicate the statistical diversity from the basal at all concentrations of atropine administered.

Atropine potently inhibited the methacholine-induced bladder contractions. The IC_{50} for atropine was 5.2×10^{-9} M and, at 10^{-7} M, only trace contractions occasionally remained (−98 ± 2%; $p < 0.001$; $n = 6$) (Figure 32).

In the resemblance with the in vivo data, atropine potently inhibited the cholinergic responses. Likewise to the previous data, obidoxime showed a dual effect that was particularly obvious in the heart preparations. The acetylcholinesterase inhibition by obidoxime seemed to of similar magnitude in the bladder and the atria. However, the antimuscarinic effect was substantially larger in the atria, which thus support the idea of obidoxime exerting a muscarinic M2 receptor antagonism.

5.3. *In vitro* effects by different doses of obidoxime on atrial frequency and urinary bladder contractions II

Methacholine evoked concentration-dependent contractions in isolated detrusor strip preparations. The maximum response was 27±2 mN (n=7) and occurred, according to the data simulation, in response to 0.5 mM of methacholine (EC50: 4.3 µM; Fig 33).

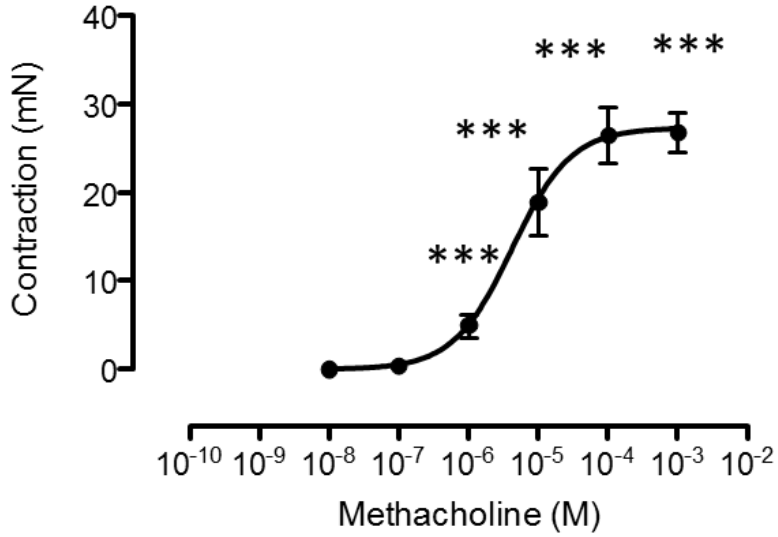


Figure 33. *In vitro* bladder contractile responses to methacholine at increasing concentrations (n = 7),

The response to methacholine at 5 μM (chosen as a standard concentration for repeated administrations; a separate series of experiments; Fig. 34) was 21±1 mN (n=6). The response was step-wise reduced in the presence of increasing concentrations of atropine (10-10 – 10^{-6} M). At 1 μM of atropine, the methacholine-evoked contraction was abolished and the IC50 value for the antagonist was 0.02 μM.

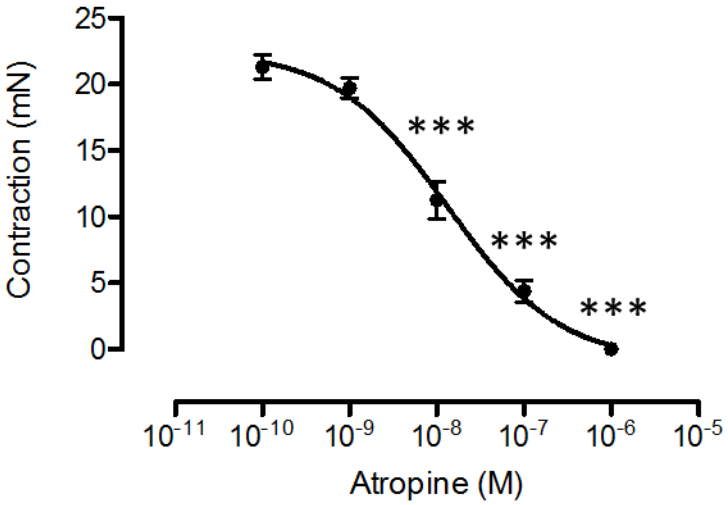


Figure 34. *In vitro* bladder contractile responses to a standard methacholine concentration (5 $X10^{-6}$ mol/L) at increasing concentrations of atropine (n = 7),

The examination of methoctramine (1 μM) inhibitory effects revealed no effect on the methacholine-evoked responses (the maximum 29.6±1.9 vs 28.4±1.4 mN; n=6; Fig. 35).

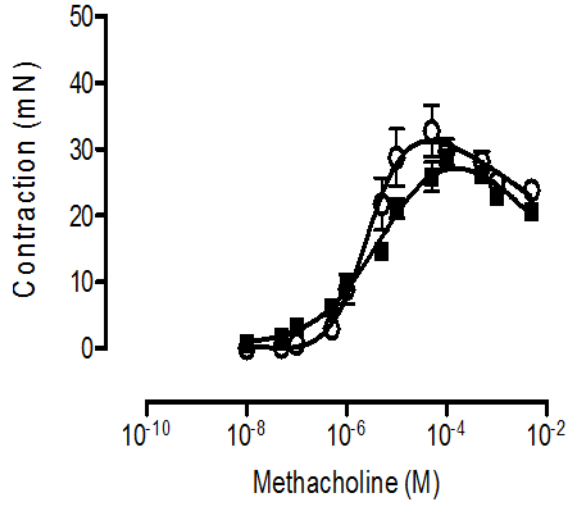


Figure 35. *In vitro* bladder contractile responses to methacholine at increasing concentrations in the absence (○) and presence (■) of methoctramine (1 lmol/L, n = 6). The vertical bars represent the SEM. ***P < 0.001.

In the atrial preparation, the basal beat frequency was 285±5 bpm (n=7), which was raised by the addition of adrenaline (10^{-4} M, 312±7 bpm; employed in order to magnify the effect of methacholine administration). Methacholine evoked concentration-dependent inhibition of the atrial frequency. The maximum inhibition occurred at 1 mM of methacholine (209±6 mN (n=7) and the EC_{50} for the methacholine inhibition was 1.0 µM; Fig. 36).

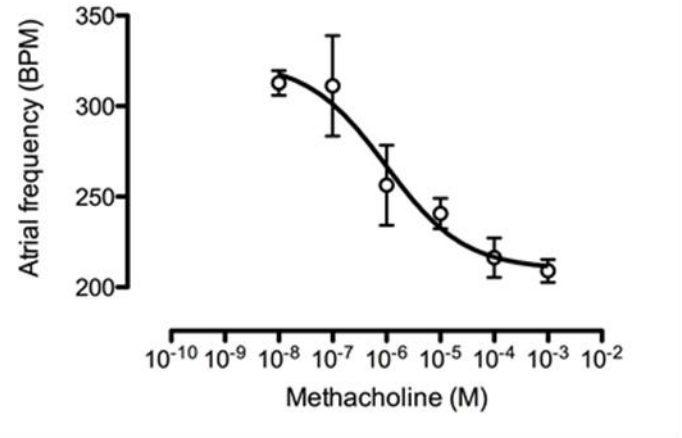


Figure 36. *In vitro* atrial frequency responses in the presence of adrenaline (104 mol/L) (a) to methacholine at increasing concentrations (n = 7),

Atropine (10^{-8} – 10^{-3} M) concentration-dependently inhibited the frequency reduction induced by the standard concentration of methacholine (1.0 µM; 287±7 bpm, n=6; Fig 37) and in the presence of 100 µM of atropine, the frequency reduction was back to the same level as

before methacholine was administered (314±6 vs 313±6 bpm; n=6). The IC_{50} value for atropine in the atrial preparations was 3.0 µM.

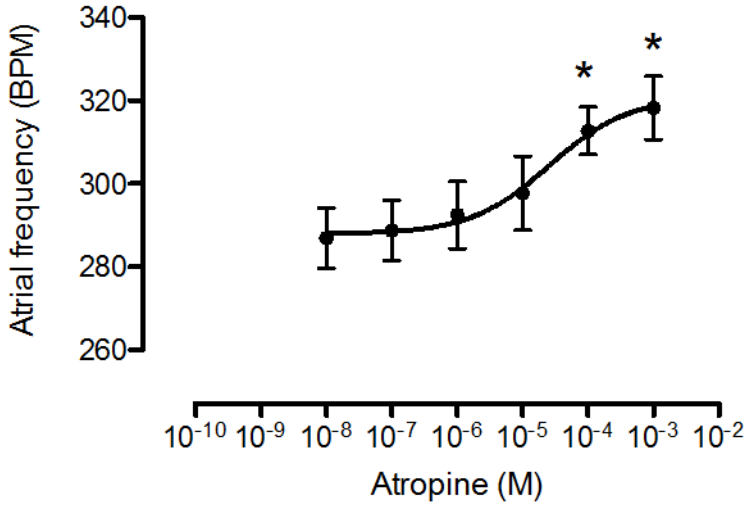


Figure 37. *In vitro* atrial frequency responses in the presence of adrenaline to a standard methacholine concentration (10^{-6} mol/L) at increasing concentrations of atropine (n = 7),

Methoctramine (1 µM) significantly inhibited the atrial frequency decrease evoked by methacholine at the concentrations of 1 µM and 10 µM (Fig. 38).

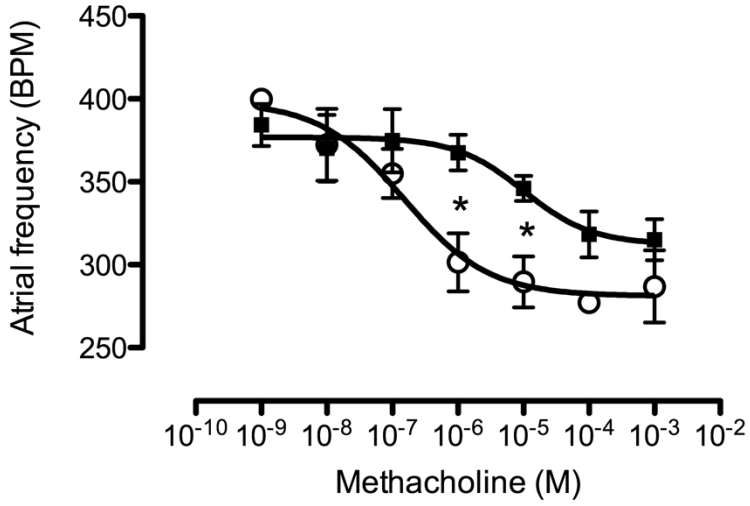


Figure 38. *In vitro* atrial frequency responses in the presence of adrenaline to methacholine at increasing concentrations in the absence (○) and presence (■) of methoctramine (1 lmol/L, n = 6). The vertical bars represent the SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Baseline tension of the bladder strip preparations was 4.7±0.4 mN (n=18). The administration of physostigmine 0.1 μM raised it by 1.5±0.5 mN (not significant vs baseline before administration of physostigmine: 5.5±1.5 mN; n=6; Fig. 38), while obidoxime 0.1 μM induced an increase by 4.7±0.7 mN (p<0.01 vs baseline before administration of obidoxime: 2.9±1.2 mN; n=6; Fig. 39).

Methoctramine (1 μM) caused a small increase of the basal tension, if anything, that did not attain significance (+0.3±0.1 mN; ns vs baseline before administration of methoctramine: 3.6±1.6 mN; n=6; Fig. 38). However, 1 μM of methoctramine in the presence of physostigmine substantially and significantly increased the basal tension (+7.7±1.6 mN, p<0.001 vs baseline before administration of physostigmine and methoctramine: 5.6±1.4 mN; n=6; Fig. 39).

Obidoxime caused a significant increase when acting together with methoctramine (+10.5±2.0 mN, p<0.001 vs baseline before administration of physostigmine and methoctramine: 3.2±1.6 mN; n=6; Fig 39).

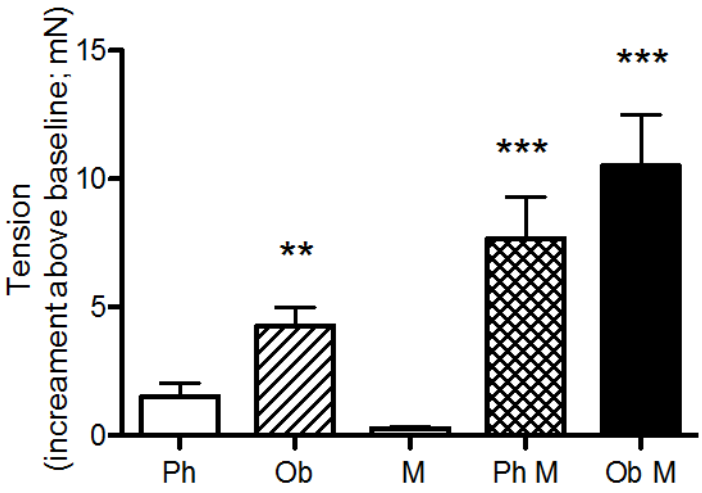


Figure 39. Increase in basal tension of urinary bladder strip preparations after administration of physostigmine (10^{-7} M; Ph), obidoxime (10^{-7} M; Ob), methoctramine (10^{-6} M; M), physostigmine (10^{-7} M) in combination with methoctramine (10^{-6} M; Ph M) and obidoxime (10^{-7} M) in combination with methoctramine (10^{-6} M; Ob M). Comparisons are made with the basal tension before administration of the compounds (Ph: 5.3±1.5 mN; Ob:

2.9±1.2 mN; M: 3.6±1.6 mN; Ph M: 5.6±1.5 mN; Ob M: 3.2±1.6 mN). The vertical bars represent the S.E.M. ** and *** denote $p<0.01$ and $p<0.001$, respectively (n=6 in each group).

Also, physostigmine possibly left-shifted the methacholine-evoked concentration-response curve as well as caused an increase of the maximum response (39.3±2.1 vs 36.6±6.4 mN; ns; Fig. 39). Similarly, methoctramine had little impact on the methacholine-evoked concentration-response curve (Fig 34). However, the combination of the two (physostigmine (0.1 μM) and methoctramine (1 μM)) significantly increased the contractile responses to low concentrations of methacholine (<5 $x10^{-5}$ M; $p<0.05 – 0.001$; Fig. 40).

In the presence of obidoxime (0.1 μM) the methacholine-evoked responses showed a different pattern than in the presence of physostigmine (Fig. 41).

At low concentrations of methacholine, obidoxime tended to increase the response in resemblance to that of physostigmine and methoctramine (Fig. 40; ns), whereas at larger concentrations of methacholine (>1 μM), obidoxime tended to decrease the contractions. In the presence of obidoxime (0.1 μM) and methoctramine (1 μM), the maximum response was significantly reduced (19.1± 2.3 vs 38.1±2.1 mN; $p<0.001$; n=6; Fig. 41).

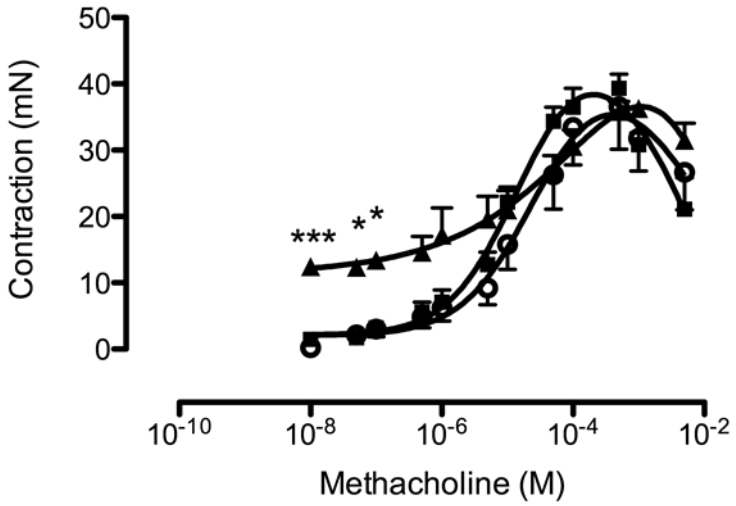


Figure 40. Methacholine-evoked *in vitro* bladder contractile responses in the absence and presence of physostigmine (10^{-7} M mol/L; ■) and physostigmine in combination with methoctramine (10^{-7} M and 10^{-6} M mol/L, respectively; ▲),

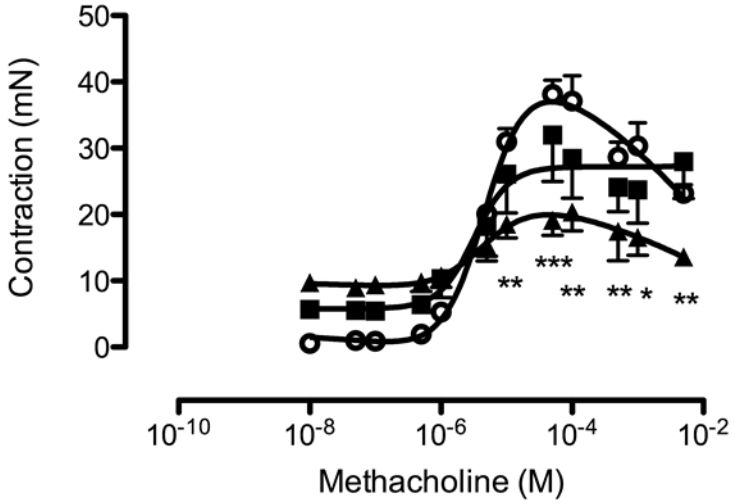


Figure 41. Methacholine-evoked *in vitro* bladder contractile responses in the absence and presence of obidoxime (10^{-7} mol/L; ■) and obidoxime in combination with methoctramine (10^{-7} and 10^{-6} M respectively; ▲). The vertical bars represent the SEM (n = 6 in each group). *P < 0.05, **P < 0.01, ***P < 0.001.

The observations on the effect of physostigmine on the responses evoked by an EC_{50} concentration of methacholine (5 and 1 μM in the bladder and atria, respectively) revealed increased responsiveness in the presence of physostigmine in the bladder preparations (Fig. 42 and 43).

In the atrial preparations, no or some enhancement of the methacholine-induced frequency decrement could possibly be observed, while obidoxime induced an increase in frequency at low concentrations (<1.5 μM). At larger concentrations of obidoxime the increments vanished.

In the bladder preparations, obidoxime induced increases at low concentrations, while at concentrations above 1 μM obidoxime inhibited the methacholine-evoked contractions. The addition of methoctramine (1 μM) at the end of the experiments did not affect the bladder response evoked by methacholine in the presence of physostigmine or obidoxime.

In the atrial preparations, however, methoctramine equalized the responses in the two groups that were treated with either physostigmine or obidoxime. Before administration of methoctramine, the atrial responses to methacholine were 66.0±12.5 and 88.4±8.1% of the basal response in the presence of 1 mM of physostigmine and obidoxime, respectively (last responses

in Fig. 42). After the addition of methoctramine (1 µM), the responses in the respective groups were 77.2±4.9 and 78.4±7.3% (ns; not shown).

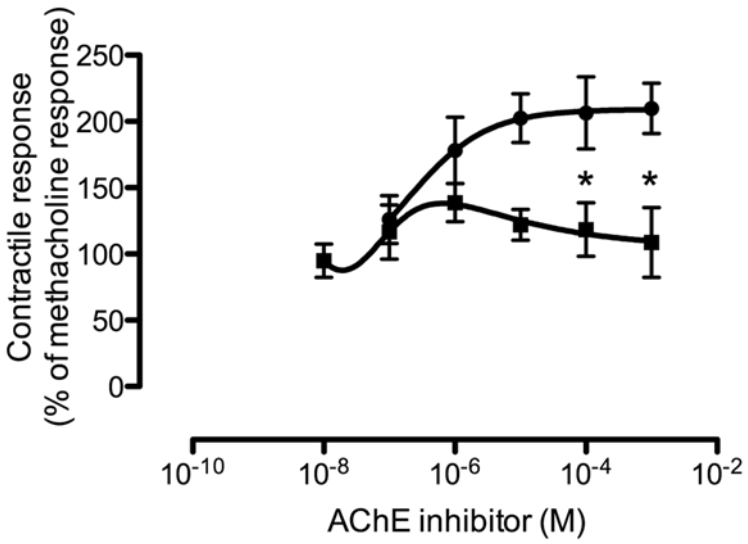


Figure 42. *In vitro* (a) bladder contractile (n = 6) and responses to a standard methacholine concentration (5 µM and 1 µM respectively. The responses are expressed as percentage of the response to methacholine at 5 µM (bladder: physostigmine group 19.9±2.0 mN; obidoxime group 18.0±2.4 mN) and 1 µM.

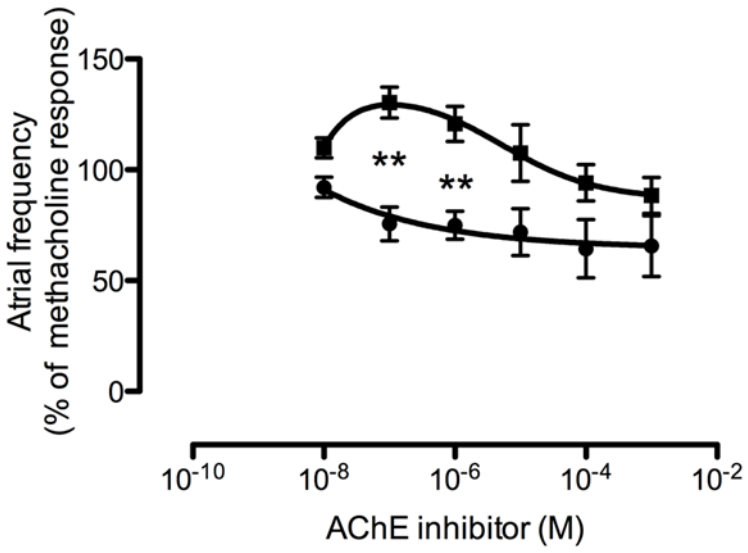


Figure 43. *In vitro* (b) atrial inhibitory (n = 4) responses to a standard methacholine concentration (5 µM and 1 µM respectively) in the presence of increasing concentrations of physostigmine (●) or obidoxime (■).The

responses are expressed as percentage of the response to methacholine at 5 μM (atria: physostigmine group 301±17 bpm; obidoxime group 298±20 bpm). The vertical bars represent the SEM. *P < 0.05, **P < 0.01, regarding comparisons between the two groups.

Thus, as based on the conclusions in the first part of this Result section, obidoxime is a weak acetylcholinesterase inhibitor that shows muscarinic M2 receptor antagonism. When comparing the effects of a pure acetylcholinesterase inhibitor (physostigmine) and of a "selective" muscarinic M2 receptor antagonist (methoctramine) with that of obidoxime, it was apparent that the combination of the first two largely mimicked the effect of obidoxime.

Furthermore, a finding of the present studies indicating a novel physiological mechanism is that muscarinic M2 receptors not only modulate β-adrenoceptor and purinoceptor effects in the urinary bladder but also muscarinic M3 receptor effects to low concentrations of acetylcholine. A certain release, just by leakage or in response to low intensity of neuronal activity of transmitter, is likely to occur. This may be a release that is not physiologically relevant. By the existence of a muscarinic M2 receptor break, such un-purposeful stimulation can by this muscarinic M2 receptor mechanism be prevented.

6. Discussion

Despite of the numerous adverse effects caused by drugs acting within the cholinergic systems, pharmaceuticals are still employed to treat diseases of gastrointestinal tract. In general, systemic administration of muscarinic receptor antagonists is more common than that of muscarinic receptor agonists which usage indication often requires only a single dose administration. A muscarinic agonist may be injected to initiate the activity of the smooth muscle in cases of atonic gut or atonic bladder. Alternative to the direct agonists, acetylcholine esterase inhibitors might be used in the treatment of glaucoma, used to overcome the neuromuscular blockade in anaesthetized patients and to initiate bladder and intestine motility in the states of atoni. One advantage of using acetylcholinesterase inhibitors is that it act in concert with the naturally occurring neuronal activity, a strategy employed for stimulating salivary secretion (Hedner et al 2001).

Another area in which drugs affecting cholinergic systems may be appropriate is when the acetylcholine esterases are blocked. Chemical warfare and pesticides have this mechanism of action, i.e. by strong and irreversible binding of esterase inhibitors to the enzyme, causing fatal by respiratory arrest, which may also be an indication. Treatment of esterase inhibition by esterase reactivators as an additive to muscarinic antagonists make the site accessible for acetylcholine by inhibiting the active site of organophosphorus (Marrs 1993).

New anticholinergic drugs were developed with a focus to exert larger effect on muscarinic M3 receptors than on muscarinic M2 receptors. This new selectivity profile has shown positive results in that less effect occurs on the prejunctional inhibitory muscarinic receptors. The selectivity on M3 over M2 receptors might even be disadvantageous. Like, if only the muscarinic M3 receptors are blocked, for instance in the respiratory tract, leaving the muscarinic M2 receptors unaffected, acetylcholine could still exert its inhibition on beta2-adrenoceptor induced relaxation.

The current thesis focuses and studies the different interaction patterns regarding muscarinic receptors subtypes. In many organs consisting of smooth muscle, the muscarinic M3 receptor causes the functional excitation; even so, muscarinic M2 receptors outnumber the muscarinic M3 receptor. This was an unsolved puzzle that lasted for years among researchers. Studies performed on muscarinic M2 receptors found that the M2 receptors indirectly contribute to the

functional responses and the functional approach was by blocking counter-acting stimuli evoked by adrenoceptors (Hegde et al 1997) and by purinoceptors (Giglio et al 2005). While the classical location of M2 receptor is heart, here, the M2 receptors on activation regulates the heart rate and affects the electrical impulses conduction. Heart regulation occurs by altering the sinoatrial node electrical activity whereas the effect on the conduction of electrical impulses is exerted through atrioventricular node. The electrical and mechanical activity of the atria and ventricles is regulated by M2 receptors. In general all the five subtypes are expressed in the tissues containing muscarinic receptors.

Muscarinic receptor expression differs between organs and also differs in the structures within the same organ. For instance, muscarinic receptors expression and signalling pathways are segment dependent in the horse respiratory tract (Abraham et al 2007). In human peripheral blood lymphocytes M3/M5 predominates M4/M2 (Tayebati et al 2001) and may participate in effecting the immune functions in asthma since they express in airway hyper-responsiveness (Ricci et al 2002). M1 or M3 receptors can cause vasoconstriction in vascular smooth muscle cells in the absence of endothelium (Ryberg et al 2008). In the endothelial cells, M3 and M5 receptors can cause indirect vasodilatation.

The normal physiological function of bladder is maintained by cholinergic, adrenergic, nitrergic and peptidergic systems. Acetylcholine is the cholinergic component, which participates in bladder function. Acetylcholine is released by both neuronal and non-neuronal (with in urothelium) (Harvey 2012). Bladder voiding is the parasympathetic regulation by muscarinic receptors that activate the detrusor smooth muscle engaging several subtypes (Giglio & Tobin 2009). When acetylcholine is released from the nerve terminals in the bladder by intense neuronal activity, prejunctional muscarinic M1 receptors are facilitating further release. If the stimulation continues, the facilitating effect vanishes and is replaced by a muscarinic M4 receptor evoked inhibition of release. Postsynaptically, muscarinic M3 receptors cause contraction, while the muscarinic M2 receptors inhibit relaxatory stimuli, which supports the muscarinic M3 receptor effect. In addition urothelial muscarinic receptor also affect the composite response. So causes muscarinic M5 receptors the release of nitric oxide, which in the healthy bladder stabilizes the basal activity.

In our functional results, obidoxime caused a dual effect *in vivo*, which did not occur, or were significantly smaller, *in vitro*. *In vivo*, low doses of obidoxime inhibited the vagal effect on heart frequency, while at larger doses the low-dose obidoxime inhibition seemed to be

counteracted. *In vitro*, the latter effect was minute or absent. In the *in vitro* examination, only small contributions from endogenously released acetylcholine are likely to occur. It therefore seems plausible to conclude that the *in vivo* counteracting effect at larger doses of obidoxime was the effect of acetylcholinesterase inhibition enhancing the vagal bradycardia effect. By this it followed that a muscarinic blocking effect (increase in frequency) by obidoxime occurred at lower concentrations than the esterase inhibition. It was also noteworthy that obidoxime by itself had no effect on the atrial frequency, which coincides with the previous observations (Bernheim et al 1992). Furthermore, in the bladder, obidoxime caused no reduction of the muscarinic receptor evoked contraction, except at a very large concentration.

Tentatively an enhancement could have occurred caused by the esterase inhibition and then followed by the inhibition of muscarinic receptor at the larger concentration of obidoxime. Nevertheless, since the bladder muscarinic contractile effect was evoked by stimulation of muscarinic M3 receptors and the bradycardia effect by muscarinic M2 receptor (Caulfield & Birdsall 1998) the present results seem plausible.

Obidoxime appears to have a greater affinity for the M2 sub-type than for the muscarinic M3 receptor sub-type. Here, obidoxime seemed to be conspicuously less potent in the atria in comparison with atropine than in the bladder. In order to magnify the effect of a muscarinic blockade, adrenaline was present during the *in vitro* experiments on the atrial preparations. Due to this manipulation, the effect of the administration of methacholine was more obvious.

Also *in vivo*, the antagonistic effect was enhanced. In this case the vagal nerve was stimulated electrically. Since vagal afferent stimulation reduces the heart rate in the anesthetized rat (Saleh et al 2001), the vagal nerve was cut coronally to the point of stimulation. This procedure caused a dramatic reduction of the stimulation-induced bradycardia, thus confirming the significant vagal afferent contribution. However, the elimination of afferent effects reduced variations in the results, probably by reducing the impact of compensatory mechanisms.

Regarding muscarinic M2 receptor selective ligands, an allosteric type of binding seems to be one reason for selectivity on this sub-type. Tentatively, the obidoxime 'M2-selectivity' might also be due to such binding characteristics, particularly in light of the relations between atrial and bladder blocking effects of atropine in comparison with that of obidoxime. Namely, while there was a huge difference regarding atropine inhibition of atrial and bladder responses, the difference was conspicuously smaller for obidoxime. Literally, atropine has been shown to

have a more potent inhibitory effect in the bladder than in the atria (Melchiorre et al 1987), however, not as great as currently.

The current experimental set-ups might explain the great differences in the potencies regarding atrial (M2 receptors) and bladder (M3 receptors) responses. In the bladder the effects of the compounds were examined on methacholine-evoked contractions and, further, almost no spontaneous activity occurred. In isolated bladder preparations, a total inhibition of the methacholine-evoked responses was thus possible to achieve without any substantial influence by differences in the basal activity of the preparations. However, in atrial preparations the situation was quite different. Firstly, there might be differences in the basal rate. Secondly, this might vary during the experiment, and, to the last, the effect of methacholine on the heart frequency was not as prominent, in a relative perspective, as it was on the bladder.

Thus, the assessment of the antagonistic potency was more un-precise in atria than in the bladder. Nevertheless, this was valid for both compounds, and the difference in potency in the two organ models was much smaller for obidoxime than for atropine.

Inhibition of the autonomic functions on the peripheral nervous system is a typical effect of muscarinic M2 receptors (Brown 2010). The classical example is to inhibit the heart frequency by vagus nerve (Schwartz et al 1992). M2 muscarinic receptors outnumber the M3 muscarinic receptors in the smooth muscle present in the urinary bladder, in respiratory tract etc.(Abraham et al 2007, Wang et al 1995). Giglio and Tobin suggested that M2 muscarinic receptors favour's the smooth muscle contraction (M3 muscarinic receptor stimulation effect) by inhibiting the relaxations which are caused by the purinoceptors (Giglio & Tobin 2009).

In the present thesis work, we focused on another interesting and important function of M2 muscarinic receptors. At lower concentrations of acetylcholine, M2 muscarinic receptors probably inhibit the contraction of smooth muscle, which in turn might prevent the urothelial accidental transmitter release induced contractions. Anticholinergic drugs (e.g. Atropine) were used along with acetylcholinesterase reactivators (oxime reactivators) *in situ* of the acetylcholinesterase inhibition by the organophosphate poisonings, carbamates, pesticides etc.(Marrs 1993).

Obidoxime, a bispyridium compound has shown the effects in a manner that it possesses antimuscarinic property and also selectivity towards muscarinic M2 receptors (Soukup et al 2013). However, no functional comparison studies have been performed with the effect of a classical esterase inhibitor. Moreover, there were no attempts made to functionally establish the

significance of the muscarinic M2 receptor antagonism occurring simultaneously to the esterase inhibition. Methacholine was currently employed as an agonist for muscarinic receptor stimulation. In the lower urinary tract, the golden standard agonist in these types of experimentations is carbachol. However, carbachol is not a pure muscarinic agonist but also acts on nicotinic receptors (Sine & Taylor 1980), likewise to acetylcholine. In order to avoid this nicotinic effect, methacholine was used. Another advantage added with this approach is its resistance towards breakdown by esterase (Bruning et al 1996).

Thus, any effect of the esterase inhibition on methacholine-evoked responses would be indirect and due to the release of endogenous acetylcholine from nerve terminals or, tentatively in the case of the bladder, from the urothelium. Muscarinic receptor antagonists exhibits various selectivity profiles (Eglen & Nahorski 2000). Methoctramine was currently used as a muscarinic M2 receptor-selective antagonist. For most of the selective muscarinic receptor antagonists the "selectivity window" is very narrow, and this is valid for methoctramine as well. Methoctramine might act in different mechanisms based on the concentrations used (Daeffler et al 1999). Previous studies displayed the discrimination between muscarinic M2 and non-M2 receptor effects, at the currently used working concentrations (Tobin 1995, Zhou et al 2002). However, while the examination of the methoctramine effects revealed an inhibitory effect on methacholine-evoked atrial responses (M2), methoctramine had no effect on the bladder preparations (M3). Thus, the methoctramine effects observed currently most probably reflect a muscarinic M2 receptor antagonism. This is in accordance with previous observations (Andersson et al 2012).

The thesis project addressed the substances' functional effects on muscarinic receptor responses. In this sort of typical studies, it is not only the ligand-receptor interaction that is important criteria for the effect variable, but also the responsiveness of the tissue that may influence the response. In studies of this similar, the bladder model seemed to be more robust than the atrial model, as known from the larger deviations of the responses indications. So, a direct comparison between the models should be made with caution; e.g. atropine potency differed markedly. Even more, comparisons of the effects of the different substances in the very same model were reliable.

In this thesis project, particularly the antimuscarinic M2 effect of obidoxime was apparent in the atrial preparations. The dual effect noticed in the urinary bladder experiments, and here the antimuscarinic effect occurred at larger obidoxime concentrations probably reflecting an

unspecific antimuscarinic effect (Fig. 41 and 42). Physostigmine, on the other hand, enhanced the cholinergic responses without showing any inhibitory effect. Also in other studies, obidoxime exerted a weak acetylcholinesterase inhibitory potency (Soukup et al 2010a). In a suggestion of a negative correlation between acetylcholinesterase inhibition and muscarinic receptor antagonism, obidoxime belongs to the group of acetylcholinesterase reactivators that exert a relatively marked muscarinic antagonism (Soukup et al 2011a, Soukup et al 2010b). Previously, the inhibition of muscarinic receptor effects by obidoxime, demonstrated to be particularly obvious in the heart (Soukup et al 2010b), which was strengthened and similes with the present results (Fig 41 and 42).

Furthermore, in the bladder the obidoxime-evoked esterase inhibition was followed by a certain antimuscarinic effect. This antimuscarinic effect is likely to reflect unselective muscarinic receptor antagonism, with a reason that the bladder muscarinic contractile effect is evoked by stimulation of muscarinic M3 receptors. Methacholine via muscarinic M2 receptors evoked the bradycardia effect (Caulfield & Birdsall 1998).

The current data indicate, also in accordance with previous observations, that obidoxime has greater affinity for the M2 subtype than for the muscarinic M3 receptor subtype. The muscarinic M2 receptor-selective profile of obidoxime is currently also indicated by the fact that methoctramine inhibited the methacholine-induced atrial frequency decrease in the presence of physostigmine but not in the presence of obidoxime. Also, obidoxime effects on basal bladder tension and on methacholine-evoked (at low concentrations) contractions showed resemblance with the combination effects of physostigmine and methoctramine.

In the methacholine concentration-response experiments, physostigmine possibly tended to cause a small increase of the contractions, if anything, at large concentrations of methacholine. At lower concentrations, no effect was observed. However, when physostigmine was added in the presence of methoctramine, baseline was increased as well as the responses to low concentrations of methacholine. Obidoxime, on the other hand, increased by itself the baseline and tended to increase responses to methacholine at low concentrations. Methoctramine further enhanced these obidoxime effects. The interpretation must be that there exists a release of small amounts of acetylcholine from some endogenous source. This is normally too low to affect the tension of the bladder strips. Not even in the presence of esterase inhibition did the release result in a contractile response. However, in the presence of methoctramine at a concentration that did

not affect the muscarinic M3 receptor-evoked contraction, the esterase inhibition resulted in tension and contraction increases.

In view of the profile of the muscarinic receptor antagonism, and the large number of muscarinic M2 receptors occurring in the bladder, a methoctramine M2 blockade is likely. This is further strengthened by the obidoxime effect since this substance thus also shows a muscarinic M2 receptor selective profile.

The current study adds new knowledge about cholinergic transmission that is important to consider when developing new entities designed to treat acetylcholinesterase inhibition. It also provides a deeper insight to the obidoxime mechanism of action, which is still considered to be one of the golden standard compounds among the oxime reactivators.

While the muscarinic M2 receptor antagonism is beneficial according to intoxication effects in the heart, the antagonism may be of disadvantage in the urinary bladder. In the bladder, the blockade of the muscarinic M2 receptor may open up for enhanced cholinergic contractions via muscarinic M3 receptors, tentatively by counteracting the potassium-induced hyperpolarization (Zholos et al 2004).

The results of the obidoxime muscarinic M2 receptor blockade would thus enhance the stimuli on the contractile muscarinic M3 receptors caused by the intoxicating esterase inhibiting substances. Since the muscarinic receptor organization in the respiratory tract shows great resemblance with that of the urinary bladder (Belmonte 2005, Giglio & Tobin 2009), the muscarinic M2 receptor antagonism may be an issue in the former organ as well (Proskocil et al 2010). In such a case, when employing compounds exerting both acetylcholinesterase inhibition and antagonism on muscarinic M2 receptors, the obstruction of the airways may be substantial and needs to be studied especially due to the fact that death caused by the acetylcholinesterase inhibitors is regarded to be a consequence of asphyxiation. Also, the current findings may explain why obidoxime is one of the most toxic reactivator (Kassa 2002).

7. Conclusions

Obidoxime is considered to be an allosteric modulator of muscarinic receptors, preferentially acting on the muscarinic M2 receptor. Obidoxime exerts muscarinic receptor inhibition and inhibition of acetylcholinesterase. At low concentrations, obidoxime exerts antimuscarinic activity, while acetylcholinesterase inhibition dominates at high concentrations.

Results of the current thesis thus depicts that oxime reactivators (obidoxime) acting on acetylcholinesterase exhibit antimuscarinic property. Interestingly, obidoxime increases the basal tension as well as responses to low concentrations of methacholine. The comparison with the classical esterase inhibitor physostigmine revealed that it does not affect the basal tension or the responses to low concentrations of methacholine. At higher concentrations of physostigmine, it tended to elevate the basal tension, likewise to that of obidoxime. However, in the presence of a muscarinic M2 receptor blockade, physostigmine induces the same pattern of response as obidoxime does.

Physiologically, these experiments demonstrate a novel and key function of the muscarinic M2 receptors. At low intensity of acetylcholine stimulation muscarinic M2 receptors exerts an inhibitory influence on the muscarinic M3 receptor. Thus a passive leakage of acetylcholine will not evolve a response. This discovery may turn out to be of central importance when new drugs acting on the acetylcholine esterase are developed.

The common cause of death in intoxications by esterase inhibitors is respiratory arrest. The treatment with reactivators thus may aggravate the risk mediated by the blocking of muscarinic M2 receptors.

8. Supplements

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