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MUSCARINIC TOXINS:

Characterization of adrenoceptor binding and applicability of membrane anchoring via glycosylphosphatidylinositol tail

MUSCARINIC TOXINS: CHARACTERIZATION OF ADRENOCEPTOR BINDING AND APPLICABILITY OF MEMBRANE ANCHORING VIA GLYCOSYLPHOSPHATIDYLINOSITOL TAIL

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Cover: Three dimensional structure of muscarinic toxin 7 from the Eastern green mamba (pdb:2vlw). Structure was drawn with the PyMOL Molecular Graphics System.

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ABSTRACT

Adrenoceptors (ARs), G-protein coupled receptors (GPCRs) at the plasma membrane, respond to endogenous catecholamines noradrenaline and adrenaline. These receptors mediate several important physiological functions being especially important in the cardiovascular system and in the regulation of smooth muscle contraction. Impairments in the function of these receptors can thus lead to severe diseases and disorders such as to cardiovascular diseases and benign prostatic hyperplasia. The Eastern green mamba (*Dendroaspis angusticeps*) venom has been shown to contain toxins that can antagonize the functions of GPCRs. The most well-known are muscarinic toxins (MTs) targeting muscarinic acetylcholine receptors (mAChRs) with high affinity and selectivity. However, some reports have indicated that these toxins might also act on the α_1- and α_2-ARs which can be divided into various subtypes; the α_1-ARs to α_{1A}-, α_{1B}- and α_{1D}-ARs and α_2-ARs to α_{2A}-, α_{2B}- and α_{2C}-ARs.

In this thesis, the interaction of four common MTs (MT1, MT3, MT7 and MTα) with the adrenoceptors was characterized. It was also evaluated whether these toxins could be anchored to the plasma membrane via glycosylphosphatidylinositol (GPI) tail. Results of this thesis reveal that muscarinic toxins are targeting several α-adrenoceptor subtypes in addition to their previously identified target receptors, mAChRs. MTα was found to interact with high affinity and selectivity with the α_{2B}-AR whereas MT7 confirmed its selectivity for the M_1 mAChR. Unlike MTα and MT7, MT1 and MT3 have a broad range of target receptors among the α-ARs. All the MTs characterized were found to behave as non-competitive antagonists of receptor action. The interaction between MTα and the α_{2B}-AR was studied more closely and it was observed that the second extracellular loop of the receptor functions as a structural entity enabling toxin binding. The binding of MTα to the α_{2B}-AR appears to be rather complex and probably involves dimerized receptor. Anchoring MTs to the plasma membrane did not interfere with their pharmacological profile; all the GPI-anchored toxins created retained their ability to block their target receptors.

This thesis shows that muscarinic toxins are able to target several subtypes of α-ARs and mAChRs. These toxins offer thus a possibility to create new subtype specific ligands for the α-AR subtypes. Membrane anchored MTs on the other hand could be used to block α-AR and mAChR actions in disease conditions such as in hypertension and in gastrointestinal and urinary bladder disorders in a cell-specific manner and to study the physiological functions of ARs and mAChRs *in vivo* in model organisms.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred in the text as roman numbers I-IV.

I) **Koivula K.**, Rondinelli S. and Näsman J. (2010) *Toxicon* 56: 440-447. The three-finger toxin MTα is a selective α_{2B}-adrenoceptor antagonist.

II) **Näreoja K.** and Näsman J. (2012) *European Journal of Pharmacology* 638: 63-70. Characterisation of α_{2B}-adrenoceptor ligand binding in the presence of MTα and delineation of structural features of receptor binding specificity.

III) **Näreoja K.**, Kukkonen J.P., Rondinelli S., Meriluoto J. and Näsman J. (2011) *British Journal of Pharmacology* 164: 538-550. Adrenoceptor activity of muscarinic toxins identified from mamba venoms.

IV) **Näreoja K.**, Louhivuori LM., Åkerman KE., Meriluoto J., Näsman J. (2012) *Biochemical and Biophysical Research Communications*. 471: 93-97. Glycosylphosphatidylinositol (GPI)-anchoring of mamba toxins enables cell-restricted receptor silencing.

CONTRIBUTION OF THE AUTHOR

The work was designed, conducted and manuscripts prepared by Katja Näreoja under the supervision and help of Dr. Johnny Näsman with the following exceptions:

I) The α_1-AR cDNAs were subcloned from a modified pOPRSVICAT into FastBac1 and baculoviruses generated with the Bac-to-Bac system by Dr. Johnny Näsman.

II) Primer design was carried out by Dr. Johnny Näsman.

III) Rat preparations were done with the help from Dr. A-C Engblom. β-adrenoceptor cDNAs were subcloned into FastBac1 and baculoviruses generated with the Bac-to-Bac system by Dr. Johnny Näsman.

IV) Toxins used in this study were constructed and produced by Dr. Johnny Näsman.

ADDITIONAL PUBLICATIONS

List of additional publications which are not included in the thesis but in which an author or co-author position obtained during PhD studies.

Rondinelli S., **Näreoja K.** and Näsman J. (2011) *Toxins* 3: 1393-1404. Molecular conversion of muscarinic acetylcholine receptor M_5 to muscarinic toxin 7 (MT7)-binding protein.

Näreoja K. and Näsman J. (2012) *Acta Physiologica* 204:186-201. Selective targeting of G protein-coupled receptor subtypes with venom peptides.

Näreoja K., Åkerman KE., Näsman J. (2012*) Biotechnology and Applied Biochemistry*. 59: 314-321. Enhanced early expression of membrane receptors with the Rous sarcoma virus promoter in baculovirus-infected insect cells.

DEFINITION OF THE PHARMACOLOGICAL TERMS USED

B_{max} – The total amount of receptor binding sites present in a cell. Usually presented as mol/mg protein.

EC_{50} – The concentration of an agonist that induces a response which is 50% of the maximal response.

Efficacy – The ability of a ligand to produce the response in the tissue. An antagonist has zero efficacy.

IC_{50} – The concentration of an inhibitor where the receptor response or binding of a ligand to the receptor is reduced by 50%.

K_d – The equilibrium binding constant. The radioligand concentration needed to achieve a half-maximum binding at equilibrium conditions. Usually presented as nM.

K_i – Inhibition constant. Usually presented as nM.

Potency – A measure of ligand activity expressed in terms of the amount required to produce an effect of given intensity.

ABBREVIATIONS

αBTx – α-bungarotoxin
ACh – Acetylcholine
AR – Adrenoceptor
BPH – Benign prostatic hyperplasia
Ca^{2+} – Calcium
$[Ca^{2+}]_i$ – Intracellular calcium level
cAMP – Cyclic adenosine monophosphate
CNS – Central nervous system
C-terminus – Carboxyl terminus (of polypeptide)
DAG – Diacylglycerol
ECL – Extracellular loop
GABA – Metabotropic γ-aminobutyric acid B receptor
GDP – Guanosine diphosphate
eGFP – Enhanced Green Fluorescent Protein
GPCR – G-protein coupled receptor
GPI – Glycosylphosphatidylinositol
GTP – Guanosine triphosphate
^3H – Tritium
IP_3 – Inositol (1,4,5) triphophate
mAChR – Muscarinic acetylcholine receptor
MT – Muscarinic toxin
nAChR – Nicotinic acetylcholine receptor
NMS – N-methylscopolamine
N-terminus – Amino terminus (of polypeptide)
PCR – Polymerase chain reaction
QNB – Quinuclidinyl benzilate
RSV-promoter – Rous Sarcoma Virus promoter
Sf – Spodoptera furgiperda
TFT – Three-finger toxin
TM – Transmembrane domain

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Katja Näreoja

1 REVIEW OF THE LITERATURE

1.1 Introduction

Plasma membrane contains numerous receptors which bind specific extracellular signaling molecules. These extracellular signaling molecules change the function of the cell and direct the cell for example to divide, change its gene expression, move or die. Molecules binding to a receptor are called ligands and can be peptides, hormones, neurotransmitters, pharmaceutical drugs and toxins. G-protein coupled receptors (GPCRs) form the largest group of membrane receptors found in the human genome. These receptors traverse the plasma membrane seven times and are arranged into a tight helical bundle which resembles a barrel when viewed from the cytoplasm.

The GPCRs can be classified into several families based on their sequences, structures and ligands that they bind. The adrenoceptors (ARs) and muscarinic acetylcholine receptors (mAChRs) covered in this thesis belong to the rhodopsin family of GPCRs. These receptors mediate a wide variety of important biological responses both in the peripheral tissues and in the central nervous system such as increases/reductions in heart rate, contraction of smooth muscle and glandular secretion. In addition to the endogenous ligands of ARs (noradrenaline and adrenaline) and mAChRs (acetylcholine), nature provides compounds that are targeting these receptors. Of these compounds many are present in the venom of animals. Several venom components act on nervous system while some disrupt cell membranes and behave as hemolytic or cytolytic peptides. The venom of mamba snakes contains muscarinic toxins which are known to bind with high affinity and selectivity to the mAChR subtypes. Studies described in this thesis were performed to characterize muscarinic toxin binding to ARs and thus to provide new tools to study adrenoceptor physiology.

1.2 The super-family of G-protein coupled receptors

Receptors at the surface of the cell bind external signaling molecules and direct these signals into the cell which eventually leads to changes in the cell's function. The receptive mechanism and the receptors were first introduced by John Newport Langley at the beginning of the 20th century (1, 2). In his pioneering study, Langley defined the receptors in the context of the action of nicotine and curare on neuromuscular transmission. Since Langley's proposition of the receptive mechanism, the receptor concept has evolved considerably and currently the International Union of Basic and Clinical Pharmacology provides extensive classification of various receptors (Classification of receptors available at http://www.iuphar.org/, site visited 17.6.2013). Generally receptors can be divided into four

distinct receptor super-families of which G-protein coupled receptors (GPCRs) form by far the largest group of membrane receptors found in humans; they comprise almost 4 % of the size of the genome accounting approximately 800 different receptors (3).

The GPCRs are classified into various families based on their amino acid sequences, structures and on the ligands that bind to these receptors (3-6). The first and the most commonly used classification system was introduced by Kolakowski in 1994 (5). This system covers GPCRs from both vertebrates and invertebrates and divides GPCRs into six classes, A-F (5). Class A is the most diverse one and contains receptors similar to rhodopsin (4, 5). Class B contains secretin and secretin-like receptors and class C glutamate receptors. Classes D, E and F contain receptors that are not expressed in humans. More recently, Fredriksson and co-workers collected ~ 800 human GPCR sequences and performed multiple phylogenetic analyses (3). Based on the sequence similarities within the transmembrane domains, the human GPCRs were divided into five distinct families; glutamate (G, n = 15), rhodopsin (R, n = 241 non-olfactory, total of 701, corresponding to family A in A-F classification system), adhesion (A, n = 24), Frizzled/Taste 2 (F, n = 24) and secretin (S, n = 15). This classification system is known as the GRAFS and is based on the initials of the family names. The rhodopsin family can be further divided to four groups (α, β, γ and δ) and 13 branches. The GPCRs covered in this thesis, the ARs and the mAChRs, belong to the rhodopsin α-family together with other biogenic amine receptors.

1.2.1 Structure of G-protein coupled receptors

G-protein coupled receptors respond to a wide variety of extracellular signals, such as hormones, neurotransmitters, smell and taste molecules, small peptides and proteins, ions and even photons. However, despite the vast diversity of ligands binding to GPCRs, these receptors share the same structural organization within the plasma membrane. GPCRs contain seven hydrophobic membrane spanning α-helical domains (TMI-VII) with the amino terminus (N-terminus) at the extracellular face of the plasma membrane and the carboxyl terminus (C-terminus) at the cytosolic part (Figure 1A). Within the plasma membrane, the highly conserved TM domains of a GPCR are arranged into a tightly packed helical bundle, a structure resembling a barrel when viewed from the cytoplasm (Figure 1B). The TM domains are connected to each other by three extracellular and three intracellular loops which have important roles in binding of ligands and in signal transduction, respectively. Conserved cysteine residues in the N-terminus and extracellular loops further stabilize the structure by forming disulfide bonds (7).

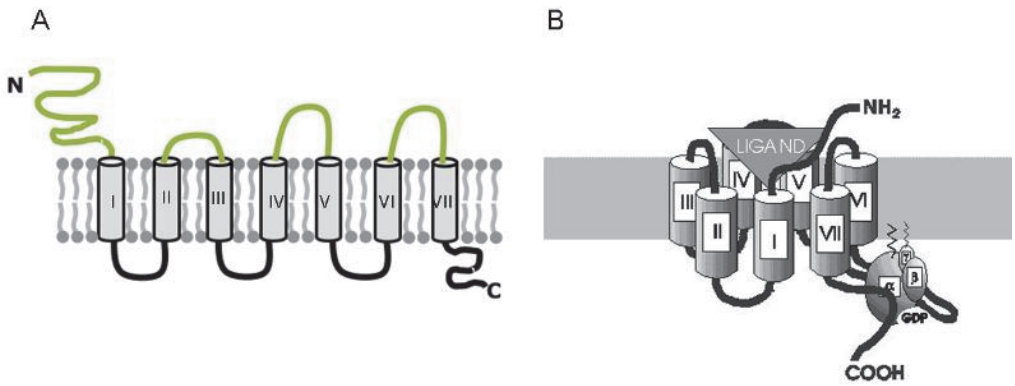

Figure 1. Structure of a G-protein coupled receptor. A. Snake diagram of a GPCR embedded in a lipid bilayer. Transmembrane domains are presented as roman numbers I-VII. The extracellular loops are presented in green, the intracellular loops in black and the amino (N) – and carboxyl (C) –terminus as letters. B. The transmembrane domains (I-VII) are arranged within the plasma membrane in a counterclockwise manner. Shown are the N- and C-terminus, the seven transmembrane domains (I-VII), the extra- and intracellular loop structures and the binding sites for ligands and heterotrimeric G-proteins. Upon ligand binding TM5 and TM6 undergo conformational changes which allow Gα subunit of the heterotrimeric G-protein to interact with the receptor (8, 9).

Initially, the general structure of GPCRs was predicted based on sequences and on hydrophobicity analyses which indicated the presence of seven TM domains connected by extra- and intracellular loops (10). The TM domains were shown to be highly conserved among the GPCRs while the N- and C-terminus and the intracellular loop connecting TMV and VI were observed to show greatest diversity (11). The first structure of a seven transmembrane protein, bacteriorhodopsin, was solved in 1990 using electron cryo-microscopy (12). Although bacteriorhodopsin is not coupled to a G-protein, its structure was used as a template to build models of GPCRs. The first crystal structure obtained of a GPCR was that of rhodopsin in 2000 (13). Thereafter, crystal structures for several GPCRs have been solved which have confirmed the predicted arrangement of GPCRs within the plasma membrane (8, 13-27). Considering the receptors covered in this thesis, crystal structures are known for the β_1- and β_2-ARs and for the M_2 and M_3 mAChRs but not for the α-AR subtypes (8, 14-16, 24, 25, 28). The crystal structures have revealed only small deviations in the structures of the solved GPCRs; an example of such a difference is an additional disulfide bond in the second extracellular loop of β_1- and β_2-ARs (15, 16).

GPCRs have classically been considered as monomers. However, evidence shows that these receptors can form both homo- and heterodimers which can affect localization, pharmacology and function of the receptors (29). The formation of GPCR dimers is thought to occur prior to receptor transport to the plasma membrane and to be mediated by covalent and non-covalent interactions including both extra- and intracellular domains and transmembrane domains (30, 31). The proper dimer formation has been noticed to be especially important for the correct

transportation of the metabotropic γ-aminobutyric acid B receptor ($GABA_B$) to the plasma membrane, and also for the function of the receptor (32). In the $GABA_B$ heterodimer, $GABA_BR1$ subunit binds the agonist, but when expressed alone, it is retained in the endoplasmic reticulum (33). The $GABA_BR2$ subunit does not bind the ligand but it is responsible for the cell surface expression of $GABA_BR1$ subunit and G-protein coupling (33). A similar phenomenon has been observed with the α_{1B}-/α_{1D}-adrenoceptor heterodimers (34).

1.2.2 Function of G-protein coupled receptors

Activation of GPCRs involves binding of extracellular signaling molecules to the ligand binding cavity (35). This induces conformational changes within the TM region and on the cytoplasmic side of the receptor. These conformational changes activate the heterotrimeric G-protein which further transduces the signal to various downstream effector proteins leading eventually to changes in cellular functions such as to stimulation of gene expression or opening of ion channels.

Heterotrimeric G-proteins are composed of three subunits; α, β and γ (36). The G-proteins, named after their Gα-subunits, are classified into four major families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$) based on the sequence similarity of the Gα subunits and on the functional outcome (37). Table 1 presents the four major G-protein families and their signaling pathways. Members of one class can be further subdivided into multiple isotypes. Currently there are over 20 Gα subunits, 6 Gβ subunits and 12 Gγ subunits found in the human genome (38, 39) which can be combined to create over 1500 variants of the Gαβγ trimer. Majority of the subunits are widely expressed but some are expressed only in certain tissues. For example, the expression of $G\alpha_{olf}$ is restricted to specific neural tissue and is enriched in the neurons of the olfactory epithelium (37) whereas $G\alpha_{15/16}$ is expressed solely in hematopoietic cells (40).

Table 1. The four major families of heterotrimeric mammalian G-proteins.

Gα	Associated effector	Second messenger	Receptor examples
$G\alpha_s$	Adenylyl cyclase	cAMP (increased)	β-AR
$G\alpha_{i/o}$	Adenylyl cyclase	cAMP (decreased)	α_2-AR
$G\alpha_{q/11}$	Phospholipase C-β	IP_3, DAG (increased)	α_1-AR
$G\alpha_{12/13}$	Rho family of GTPases	--	actin cytoskeleton

cAMP-cyclic adenosine monophosphate; IP_3-inositiol (1,4,5) triphophate; DAG-diacylglycerol

The activation cycle of a GPCR – G-protein complex by an agonist is summarized in Figure 2. When the receptor is in the inactive state the subunits of heterotrimeric G-protein are associated and guanosine diphosphate (GDP) is bound to the Gα subunit. This Gα-GDP-Gβγ heterotrimer makes interactions with the receptor at the intracellular face of the plasma

membrane. Ligand binding to the receptor triggers conformational changes not only in the receptor itself but also in the Gα subunit resulting in the exchange of GDP with guanosine triphosphate (GTP) and dissociation of the heterotrimer into two functional subunits, Gα and Gβγ. Both Gα and Gβγ can bind to a wide variety of effectors and initiate signaling cascades. (41)

The third intracellular loop of the receptor seems to be the most important one in determining the interaction between the receptor and G-protein (42). For example, the β_2-ARs having two-thirds of their third intracellular loop deleted are unable to bind to effectors and to activate signaling cascades (43). Additionally, TM5 and TM6 of ligand-free rhodopsin and β_2-AR have been reported to undergo conformational changes which allow Gα subunit of the G-protein to interact with the receptor (8, 9). Besides this the second intracellular loop and the C-terminus have been shown to guide the receptor in G-protein selection and activation (44-47). The corresponding interaction domain in the G-protein appears to reside in the C-terminus of the alpha subunit but interactions sites can be found also in the N-terminus (8, 48-51). Exchange between alpha subunits of as few as five amino acids in this region allows for a switch in G-proteins receptor selectivity (52).

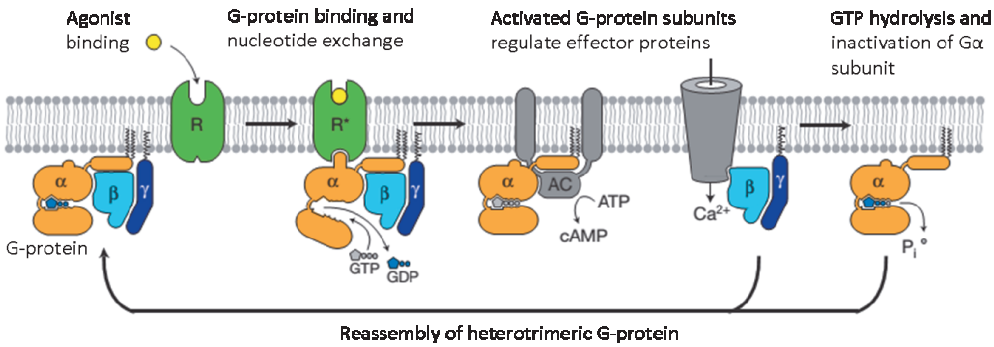

Figure 2. Activation cycle of a G-protein coupled receptor. In an inactive state, the α- and βγ-subunits of a G-protein are associated. Binding of an extracellular agonist to GPCR (R) induces conformational changes within the receptor enabling G-protein binding. In the Gα-subunit GDP is exchanged with GTP. GTP bound Gα and Gβγ dissociate and activate various downstream effector proteins such as adenylyl cyclase (AC) and Ca^{2+} channels. Intrinsic GTPase activity of Gα-subunit eventually hydrolyses GTP to GDP leading to re-association of Gα- and βγ- subunits. This together with agonist dissociation terminates the signaling. Figure is reprinted with permission from 8.

Gα subunit has intrinsic GTPase activity which hydrolyses GTP to GDP thus terminating the signaling and allowing the re-association of Gα and Gβγ subunits (53, Figure 2). Although hydrolysis of GTP to GDP terminates the signaling occurring through a particular G-protein, the receptor is still active due to the presence of agonist and can activate a new set of G-proteins. In order to adapt to the stimulus or turn off signaling completely, receptors must be

desensitized which involves phosphorylation of the receptors. Receptor desensitization can be either homologous or heterologous. In homologous desensitization the activated receptor serves as a substrate for G-protein coupled receptor kinases which phosphorylate serine and threonine residues present in the intracellular parts of the activated receptor (54). Phosphorylated receptor serves then as a sign for arrestins to bind. Arrestin binding to the activated and phosphorylated receptor blocks further G-protein binding and activation eventually attenuating the signaling (55). As a result of this, the receptor is rapidly internalized. The internalized receptor is either dephosphorylated and recycled back to the plasma membrane or directed to lysosomes for degradation. Heterologous desensitization, on the contrary to homologous desensitization, is also known as receptor activation-independent regulation and involves second messenger activated protein kinases A and C. These kinases phosphorylate and desensitize receptors even in the absence of agonists (56, 57). The receptors desensitized in heterologous manner are less able to be activated by their own agonist.

Receptor dimerization can additionally modulate the properties of GPCRs such as targeting of GPCRs to the plasma membrane, activation, G-protein coupling, signaling and internalization (29, 58, 59). Changes in signaling can be seen with the dopamine D_1-D_2 receptor heterodimer. Normally, dopamine D_2-receptor is coupled to $G_{i/o}$ leading to reduction in cyclic adenosine monophosphate (cAMP) levels. However, upon heterodimerization with the dopamine D_1-receptors, D_2-receptor becomes coupled to $G_{q/11}$. In this way heterodimerization of D_1- and D_2-receptors provides a mechanism by which dopamine can activate Ca^{2+} release through phospholipase C. (60).

1.2.3 Ligand binding to G-protein coupled receptors

Ligands acting on GPCRs can be roughly divided into three classes; agonists, antagonists and allosteric modulators. An agonist is defined as a ligand binding to a receptor and triggering a biological response. Agonists can be further divided into full, partial, and inverse agonists and they all bind to the same orthosteric binding site. Full agonists produce maximum response upon binding and thus have high efficacy. Partial agonists have lower efficacy than full agonist. They are able to activate the receptors but produce responses that are only partial of the maximum response irrespective of the concentration applied. Some receptors, such as β_2-ARs, show weak constitutive activity in the absence of ligands. Inverse agonists decrease this activity below basal level producing an opposite effect compared to full and partial agonists. (61, 62). The effects of agonists on receptor activity are illustrated in Figure 3.

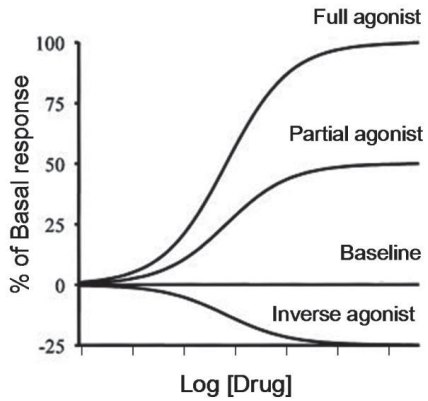

Figure 3. The effects of agonists on the activity of receptors. The baseline represents constitutive activity in the absence of agonists. The figure is based on figure 2-22 from reference 63.

Antagonists produce no biological response upon receptor binding. They function by blocking or weakening the responses created by agonists. Antagonists are either competitive (produce surmountable blocking) or non-competitive (produce insurmountable blocking). Competitive antagonists bind to the same orthosteric binding site as agonists and compete with them. These antagonists produce rightward shifts in concentration-response curves without altering the maximum response (Figure 4A). Non-competitive antagonists bind to a site distinct from the orthosteric one. They block the receptor responses and result in depression of maximum response (Figure 4B). (64). However, in many cases the non-competitive antagonism is caused by irreversibility of the ligand-receptor interaction or alternatively is a result of slowly dissociating antagonist.

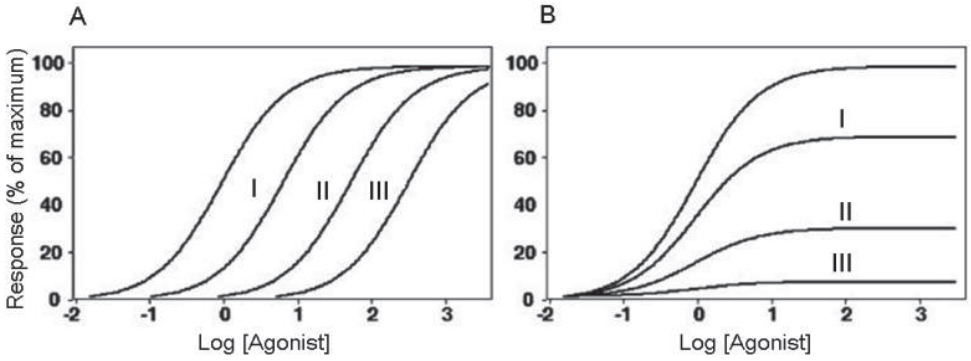

Figure 4. Theoretical concentration-response curves for receptors preincubated with antagonist. Curves on the left; no antagonist exposure. Curves with roman numbers (I-III) represent treatment with increasing antagonist concentrations. A. Competitive antagonist. B. Non-competitive antagonist. The figure is modified and reprinted with permission from Figure 1 in 64.

Allosteric modulators have an indirect effect to the binding properties of agonists by binding to a site which is distinct from the orthosteric binding site. These modulators act by changing the conformation of the receptor and thus have impact on the affinity and efficacy of orthosteric ligands (65). Classical examples of allosteric modulators of GPCRs include gallamine and alkane-bisammonium compounds such as W84 which inhibit the functions of mAChRs (66, 67). Allosteric modulators can be divided into positive, negative and neutral modulators. A positive allosteric modulator does not have intrinsic agonistic effects; it functions by amplifying the effects of orthosteric agonists. Negative allosteric modulators can be regarded as non-competitive antagonists. These modulators function by decreasing the affinity and/or effects of orthosteric ligands. Neutral allosteric modulators do not change the affinity of orthosteric ligands at equilibrium, but can inhibit the actions of other allosteric modulators binding to the same site. (65).

1.2.3.1 Orthosteric ligand binding cavity

Most small agonists bind to a cavity formed by the transmembrane domains whereas peptide hormones and proteins bind to the N-terminus and extracellular sequences connecting the TM domains (68). However, the size of the ligand does not always predict the site where it binds. For example, glycoprotein hormones, the calcium ion and glutamate all activate their respective receptors by binding to the large N-terminal domains (68).

In bovine rhodopsin the binding cavity for covalently bound 11-cis retinal is formed by TM3-TM7 and is covered with the second extracellular loop (ECL2) which folds as a β-hairpin above the cavity (13, 69). This fold has been observed to be stabilized by a disulfide bridge (13). The binding cavity of rhodopsin-like GPCRs is similar to that of rhodopsin and the residues present in TM domains 3, 5, 6 and 7 have been implicated to have important roles in ligand binding in ARs (70). However, recent evidence from crystal structures reveals that the ligand binding site in ARs involves also residues present in extracellular domains (14-16). Figure 5 illustrates the structure of turkey β_1-AR, part of its ligand binding cavity and some of the amino acids taking part in ligand binding.

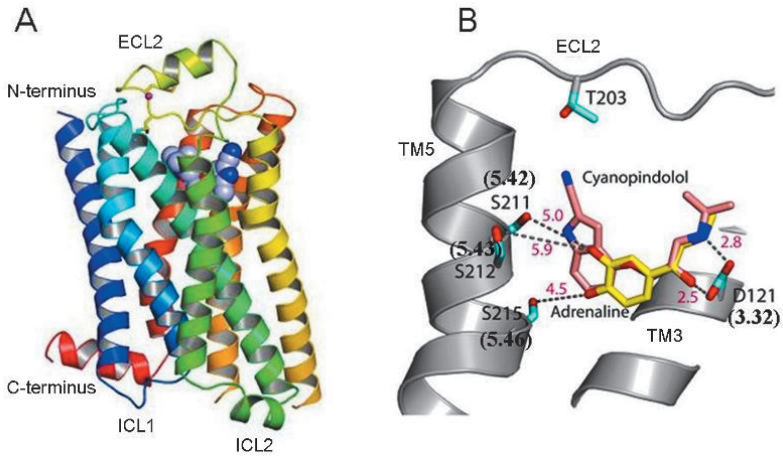

Figure 5. Ligand binding cavity of turkey β_1-AR. A. Ribbon presentation of the β_1-AR structure. Extracellular loop 2 (ELC2), intracellular loops 1 and 2 (ICL1, ICL2) and C- and N-terminus that were truncated in the crystallized structure are labelled. Shown is also natrium ion (in pink) which stabilizes the helical conformation of ELC2 and thus the structure of the entrance to the ligand-binding cavity. B. Superposition of a model of the agonist, adrenaline (yellow), with the structure of the antagonist, cyanopindolol (pink), as it binds to the β_1-AR. Shown are the TM domains 3 and 5, ELC2 and amino acids whose side chains are known to interact with the hydroxyl groups on the catechol ring of the agonist (S=Ser, T=Thr, D=Asp). The Ballesteros-Weinstein numbering of amino acids is shown in brackets. Reprinted with permission from 16.

TM domain 3 contains a highly conserved aspartate residue at position 3.32 (according to the Ballesteros-Weinstein numbering (71)) and its negatively charged side chain has been shown to provide anchoring points for ligands and to play a key role in receptor activation (70). In addition to TM3, residues in TM5 are involved in binding of classical orthosteric ligands. In the α_{2A}-AR particularly important are Ser200 (5.42), Cys201 (5.43) and Ser204 (5.46). These amino acids have been linked not only to ligand binding but also for receptor activation (72, 73). Besides the TM domains, ECL2 has been reported to participate in ligand binding in several rhodopsin-like GPCRs including ARs (16, 74, 75), adenosine receptors (76, 77), dopamine receptors (78) and mAChRs (79, 80). This loop is constrained above the ligand binding site by a disulfide bridge which is formed by cysteines at positions xl2.50 (The Ballesteros–Weinstein numbering scheme was extended to the ECL2 by Xhaard and co-workers (81)) and 3.25. The amino acids present in the ECL2 have noticed to be important for the high affinity binding and specificity of various ligands for GPCRs but also for the species-selectivity of ligands. For example, substituting amino acids in the ECL2 of α_{1A}- and α_{1B}-ARs changed their subtype selectivity for phentolamine and WB4101 (74) whereas in type-1D serotonin receptors, replacement of ECL2 resulted in a mutant whose affinity for ketanserin (selective antagonist) was approximately 46-fold lower compared to the wild type receptor (82, 83). Regarding the species-selectivity of ligands, in α_{2A}-AR, three amino acids, Arg187 (xl2.49) and Glu189 (xl2.51) from the ECL2 and Cys201 (5.43) from the TM5, were found to

determine the species selectivity of yohimbine and rauwolscine for the human and mouse α_{2A}-ARs (75).

In addition to the specific amino acids, receptor dimerization can have influences on the ligand binding and pharmacological properties. For example, heterodimerization of two fully functional opioid receptors, κ and δ, resulted in a receptor whose binding properties were different from monomeric κ and δ opioid receptors; the κ/δ heterodimer did not show significant affinity for the two highly selective κ and δ agonists and antagonists (84). On the contrary, high affinity binding was detected with partially selective κ and δ opioid receptor ligands (84). It has also been observed that a ligand specific for one receptor in a GPCR heterodimer can affect to the affinity of another ligand specific for other monomer in the heterodimer. This phenomenon is characterized by the dopamine-somatostatin heterodimer where binding affinity of somatostatin agonist somatostatin-14 is increased by 3000 % upon addition of dopamine agonist quinpirole (85). However, there are many GPCRs whose binding properties are not altered upon dimerization (86-88). For example, heterodimerization of α_1-ARs does not alter the affinity for noradrenaline, prazosin and subtype specific antagonists suggesting that heterodimer formation does not produce pharmacologically distinct subtypes (88).

1.2.3.2 Allosteric ligand binding site

The GPCRs can be considered naturally allosteric as they possess more than one type of binding site with the G-proteins being the best-known allosteric modulators of agonists binding to GPCRs. Allosteric modulators, other than the G-proteins, interact with the GPCRs through an extracellular site which is distinct from the orthosteric binding site. This binding site is also often less conserved than the orthosteric one. (89).

The mAChRs are the best studied examples of allosteric phenomena among the GPCRs. The earliest evidence of an allosteric binding site on the mAChRs was obtained from functional studies on the M_2 mAChR with gallamine and W84 (66, 67). Later on the crystal structures of GPCRs have indicated that these receptors have a large extracellular vestibule which binds the allosteric modulators (90). The allosteric binding site has been shown to be situated directly above the orthosteric binding site accounting for the fact that the ligands binding to the allosteric binding site can modulate the affinity and efficacy of orthosteric ligands (90). Closer investigations, such site-directed mutagenesis and chimeric receptor studies in addition to the crystal structures of the M_2 and M_3 mAChRs, have shown that several amino acids in the ECL2, ECL3 and TM7 take part in allosteric ligand binding in the mAChRs (28, 90, 91). For example Tyr177 in the ECL2 of the M_2 mAChR has been shown to interact with a positive allosteric modulator, LY2119620 (90). On the TM7, Trp422 has been implicated to take part in the binding of allosteric ligands in the M_2 mAChRs (28, 90). In addition to these,

some residues in ECL2 (Tyr80), ECL3 (Asn419) and TM6 (Asn410) of the M_2 mAChRs have been noticed to form hydrogen bonds with the allosteric ligand (90) whereas Glu172 present in the ECL2 has been indicated to be required for gallamine's selectivity (92).

1.3 Adrenoceptors

The adrenoceptors, classically involved in the 'fight or flight' reactions, respond to the endogenous catecholamines noradrenaline (neurotransmitter) and adrenaline (hormone) (Figure 6). Both of these catecholamines are derived from the amino acid tyrosine and belong to phenylethylamines which consist of a catechol moiety (a benzene ring with two attached hydroxyl groups) and an aliphatic amine side chain. The biosynthetic pathway leading to these catecholamines includes several enzymatic reactions with tyrosine hydroxylase as the rate limiting enzyme. (93)

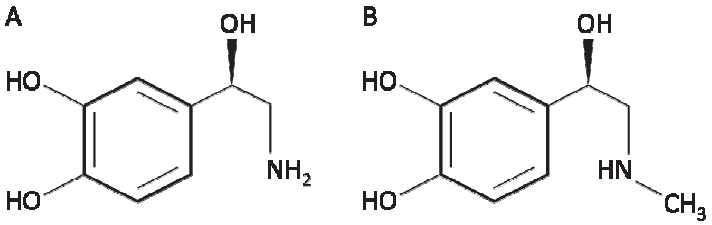

Figure 6. The chemical structure of A. noradrenaline and B. adrenaline, a methylated derivative of noradrenaline.

During the fight or flight reactions (also known as acute stress response), the sympathetic nervous system becomes activated and induces the adrenal medulla to release high levels of adrenaline and noradrenaline. In addition to adrenal medulla, noradrenaline is released from the nerve terminals of the sympathetic nervous system. Due to sudden release of catecholamines, adrenoceptors become highly activated. This results in various changes in the body including increases in heart rate and rise in blood pressure with a concomitant increase in blood flow to major muscle groups preparing the animal or man to respond rapidly to a threatening situation. The blood glucose levels are also elevated to support the energy demand. (94).

1.3.1 Classification of adrenoceptors

The primary classification of ARs into α- and β-ARs was done by Ahlquist in 1948 and was based on pharmacological criteria, i.e. the rank order by which natural catecholamines (adrenaline and noradenaline) and certain synthetic amine agonists (such as isoproterenol and α-methyl noradrenaline) induced effects on different organs (95). Among the ligands tested

noradrenaline and adrenaline were the most potent on the α-ARs whereas isoproterenol and adrenaline were the most potent on the β-ARs (95). In the 1960s the β-ARs were further subdivided into β_1-AR and β_2-AR by Lands and co-workers (96, 97). This division was based on the different potencies of noradrenaline and adrenaline at these receptor sites. The third β-AR subtype, β_3-AR, was isolated at the beginning of the 1990s (98-100). Compared to the other two β-AR subtypes, noradrenaline is more potent than adrenaline in stimulating the β_3-AR subtype (98-100).

The α-ARs were not studied thoroughly until the 1970's when they were, based on anatomy, subdivided to postsynaptic α_1-ARs and to presynaptic α_2-ARs (101). Another classification system divided them according to their functional properties i.e. α_1-ARs caused excitatory responses and α_2-ARs inhibitory responses (102). However, later it became evident that these classification systems were inappropriate with regard to α_2-ARs as these receptors were found to exist on both pre- and postsynaptic sites and in non-synaptic locations (103) and to mediate both excitatory and inhibitory functions (104).

The first AR to be cloned was the hamster β_2-AR in 1986 (105) followed by other ARs at rapid intervals (106-113). Development of cloning techniques and the different affinities of radiolabelled ligands for the receptors greatly helped to divide ARs into various subtypes. The current division of ARs is based on both the amino acid sequences and on the biological and pharmacological properties of the receptors. The major AR types are α_1-ARs, α_2-ARs and β-ARs. Each of these AR types can be further divided into three subtypes encoded by distinct genes; α_1-ARs to α_{1A}, α_{1B} and α_{1D}, α_2-ARs to α_{2A}, α_{2B} and α_{2C} and β-ARs to β_1, β_2 and β_3 (Figure 7). In addition to these subtypes, there are reports proposing a putative fourth subtype for each major receptor class (114-117).

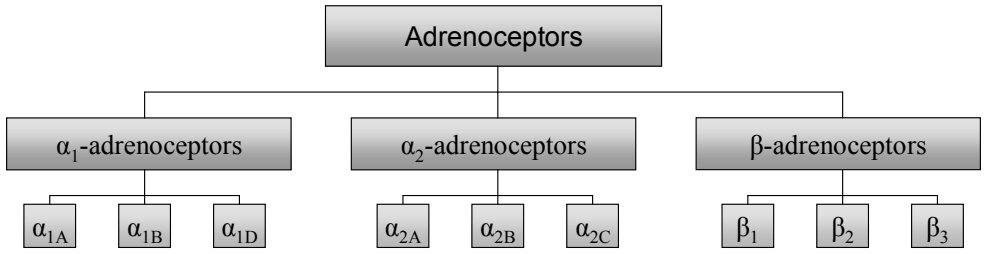

Figure 7. Classification of adrenoceptors. Shown in the figure are the adrenoceptors present in mammals.

1.3.2 Physiology

Adrenoceptors are widely distributed in the body being present both in the central nervous system (CNS) and in peripheral tissues. These receptors are involved in many physiological

functions but particularly important they are in the cardiovascular system and in the regulation of smooth muscle contraction. In the CNS, the role of AR function is less well defined, but knock-out mice are beginning to unravel their particular functions (118-120).

The three α_1-AR subtypes are considered to be stimulatory receptors which signal through the G-proteins of the $G_{q/11}$ family. Stimulation of α_1-ARs leads to activation of phospholipase C through actions of $G_{q/11}$ and subsequent generation of second messengers IP_3 and DAG by the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate on the plasma membrane. IP_3, released into the cytosol, binds to IP_3 receptors present on the smooth endoplasmic reticulum resulting in mobilization of Ca^{2+} from intracellular stores and muscle contraction.

The α_1-ARs are highly expressed in the heart and prostate but they are widely expressed also in the liver and in smooth muscle (121-123). The most important actions of α_1-ARs are contraction of smooth muscle and regulation of blood pressure (124). A sudden fall in blood pressure activates the baroreceptor reflex which further activates the α_1-ARs leading to contraction of less vital vascular beds, particularly in skin and internal organs. Besides the vital role in blood pressure control, the α_1-ARs are involved in prostate function (125). The importance of α_1-ARs in prostate function is evident in benign prostatic hyperplasia (BPH) where enlargement of prostate complicates normal urine flow. Activation of the α_1-ARs relaxes the muscles of prostate and bladder neck, making it easier for men to urinate. In addition to their high expression levels in smooth muscle and the heart, all three α_1-ARs show wide but rather restricted expression patterns in the CNS of mammalians (126, 127). In the CNS the α_1-ARs have a role in addiction (119), nociception (120) and modulation of working memory (120).

In contrast to α_1-ARs, the three α_2-AR subtypes have traditionally been reported to have mostly inhibitory roles in the body by coupling to pertussis toxin sensitive G-proteins, G_i and G_0. This interaction inhibits the opening of voltage-gated Ca^{2+} channels in neuronal cells (128, 129) and decreases the activity of adenylyl cyclase leading to reduction in cellular cAMP levels (130, 131). However, α_2-ARs have also been linked to stimulation of Ca^{2+} influx (132-135) and to activation of voltage-gated potassium channels (136).

The α_2-ARs are present in virtually every peripheral tissue (137-139). However, some tissues have been found to express predominantly only one α_2-AR subtype; for example the α_{2A} is the only detected subtype in the aorta and in the spleen whereas the α_{2B} is the only α_2-AR subtype present in the liver (140). Most of the classical effects mediated by the α_2-AR agonists are governed by the α_{2A}-AR subtype such as hypotensive and bradycardic effects (141, 142) and sedation (143). This particular subtype plays a dominant role in cardiovascular regulation as confirmed by α_{2A}-AR knockout mice which show increases in blood pressure and heart rate

(144). Compared to the α_{2A}-AR, rather few functions have been described for the other two α_2-AR subtypes. The α_{2B}-AR mediates increases in systemic blood pressure but it also seems to be important for the organ development and proper reproduction (145, 146). The α_{2C}-ARs are mainly expressed in the CNS where they modulate various behavioral responses such as aggressive behavior, startle reactivity and amphetamine induced locomotor activity (147). This particular subtype also has a putative role in stress-dependent depression (148).

All three β-AR subtypes are positively coupled to adenylyl cyclase through activation of G_s protein. This in turn results in increased levels of cellular cAMP, activation of protein kinase A and inhibition of Ca^{2+} release from intracellular stores. The β-AR subtypes show rather restricted expression in various organs and tissues being extensively distributed in the cardiovascular system and in smooth muscle (149-152). The β-ARs have for a long time been established as key players in maintaining cardiovascular homeostasis. Stimulation of β_1-ARs, the predominant β-AR subtype found in the heart (150), induces positive chronotropic (increases in heart rate) and inotropic effects (increases in force of contraction). As a consequence, these receptors are often either stimulated or blocked to treat common diseases such as hypertension, cardiac arrhythmias and ischemic heart disease. In the smooth muscle, particularly in the bronchial smooth muscle, the β_2-ARs mediate relaxation of the muscles in response to activation (153). The third subtype, the β_3-AR, is present in large amounts in the brown and white adipose tissues (98). In the brown adipose tissue the β_3-AR most likely regulates noradrenaline-induced changes in energy metabolism and thermogenesis (154). However, all the β-AR subtypes seem to be important for the regulation of metabolism as mice lacking all three β-ARs develop massive obesity (155).

1.3.3 Drugs and therapeutics

Hypertension, more commonly known as high blood pressure, is a condition where the blood pressure in the arteries is elevated increasing the risk for stroke, heart disease and death. For many years, β-AR antagonists have been used to treat hypertension (156). These drugs target mainly the β_1-ARs thereby reducing the heart rate and the contractility of the heart. The β-AR antagonists are also commonly used in other cardiovascular conditions such as in the treatment of angina pectoris, cardiac arrhythmia and for the long-term treatment of patients who have survived myocardial infarction ([156]). Additionally, these drugs have also been useful in the treatment of migraine, anxiety disorders, hyperthyroidism, alcohol withdrawal, glaucoma and ocular hypertension. In some cases the α-blockers have been used to treat hypertension (157). The α-AR antagonists, especially α_1-AR antagonists, have been useful in the treatment of primary hypertension; they show 8-10 % decrease in systolic and diastolic blood pressure (158). However, their use has not been as widespread as of the other antihypertensive drugs and nowadays the α_1-AR antagonists are no longer recommended for

the treatment of early-stage hypertension (158). Despite this, they can be added as individual agents to treat refractory hypertension (159).

Although α_1-AR antagonists are not recommended for the treatment of early-stage hypertension, BPH, a urologic disorder prevalent in elderly men, is treated mainly by α_1-AR antagonists. Alfuzosin was the first uroselective α_1-AR antagonist to be evaluated in the treatment of BPH (160) and was subsequently marketed, initially in France, in 1987. This drug became the standard α_1-AR antagonist in the treatment of BPH and is widely used in Europe. Other antagonists widely used are prazosin and tamsulosin. Prazosin can be considered the prototype of α_1-AR ligands and it displays equal affinities for all three α_1-AR subtypes (161), but it also binds to α_2-ARs (162). Tamsulosin is the only uroselective α_{1A}-AR antagonist commercially available and it does not have impact on the heart rate or blood pressure (163).

Yohimbine and rauwolscine, a stereoisomer of yohimbine, are classical α_2-AR antagonists. These compounds belong to the family of yohimbanes which are complex polycyclic structures. Yohimbine, extracted from the bark of the *Pausinystalia yohimbe* tree, is α_2-AR ligand used for the treatment of male impotence (164). Side effects of yohimbine are dose-dependent and only small increases in heart rate are observed with doses exceeding the well-tolerated level (10 mg). The first highly selective α_2-AR ligand, an antagonist JP-1302, was discovered through a high-throughput screening approach (165). This compound shows approximately 100-fold selectivity for the α_{2C}-AR over the α_{2A}-AR and α_{2B}-AR subtypes (165). It is speculated that JP-1302 would have therapeutic potential for the treatment of neuropsychiatric disorders such as depression and schizophrenia as it has been noticed to display antidepressant and antipsychotic-like effects in animals (147, 166). Another ligand showing slight subtype selectivity is MK-912 which is about 4- and 13-fold more selective for the α_{2C}-AR as compared to the α_{2A}-AR and α_{2B}-AR subtypes (167). In addition to the antagonists described, α_2-AR agonists are being used as antihypertensive drugs of which clonidine serves as an example (157), as veterinary sedatives (168) but also in the treatment of attention-deficit hyperactivity disorder (169). Table 2 lists some α-AR ligands and their subtype selectivities.

Table 2. The ligands acting on α-adrenoceptors and their binding preferences.

Ligand	Binding preference	Reference
α_1-adrenoceptor ligands		
Agonists		
Noradrenaline	α_{1A}-AR ~ α_{1B}-AR ~ α_{1D}-AR	170
Adrenaline	α_{1A}-AR > α_{1B}-AR ~ α_{1D}-AR	170
Oxymetazoline	α_{1A}-AR >> α_{1B}-AR ~ α_{1D}-AR	171
Antagonists		
Prazosin	α_{1A}-AR ~ α_{1B}-AR ~ α_{1D}-AR	161
Silodosin	α_{1A}-AR >> α_{1D}-AR > α_{1B}-AR	171
Tamsulosin	α_{1D}-AR ~ α_{1A}-AR > α_{1B}-AR	161
α_2-adrenoceptor ligands		
Agonists		
Noradrenaline	α_{2A}-AR ~ α_{2B}-AR ~ α_{2C}-AR	170
Adrenaline	α_{2A}-AR ~ α_{2B}-AR ~ α_{2C}-AR	170
Clonidine	α_{2A}-AR > α_{2B}-AR > α_{2C}-AR	172
UK-14,304	α_{2A}-AR >> α_{2C}-AR > α_{2B}-AR	172
Antagonists		
JP-1302	α_{2C}-AR >> α_{2B}-AR ~ α_{2A}-AR	165
MK-912	α_{2C}-AR > α_{2A}-AR ~ α_{2B}-AR	173
RX-821002	α_{2A}-AR ~ α_{2B}-AR ~ α_{2C}-AR	174
Yohimbine	α_{2C}-AR > α_{2A}-AR > α_{2B}-AR	175

1.4 Muscarinic acetylcholine receptors

Acetylcholine (ACh) was first identified in 1914 by Henry Dale (176) but its role as a transmitter was not discovered until 1930s by Dale and co-workers. ACh functions in the peripheral and central nervous systems and activates two distinct classes of plasma membrane receptors; muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs) (176). The mAChRs were named after muscarine, a hallusinogenic alkaloid from

mushrooms, whereas the nAChRs, ligand-gated ion channels located on neurons and on the postsynaptic side of the neuromuscular junction, were named after nicotine found from nightshade family of plants (62).

Currently there are five muscarinic acetylcholine receptors (mAChR), M_1-M_5, which have been cloned and whose pharmacological profiles have been determined (177). These receptors mediate most of the excitatory and inhibitory actions of ACh in the central and peripheral nervous systems and can be divided into two different classes based on the G-proteins to which they are coupled. M_1, M_3 and M_5 mAChRs couple to the G-proteins of the $G_{q/11}$ family which results in increases in IP_3 levels and in intracellular calcium levels (178). M_2 and M_4 mAChRs preferentially couple to $G_{i/o}$ type G-proteins leading to a decrease in the activity of adenylyl cyclase and modulation of ion channel activity (178).

As with ARs, the mAChRs are widely distributed in the body although each receptor subtype exhibits its own distinct distribution patterns both in the CNS and in peripheral tissues (179). Regarding the distribution of mAChRs in different organs and tissues, only a few examples will be mentioned in this context. For example, M_1 and M_4 mAChRs are the most abundant subtypes in the CNS (179). In the peripheral tissues it is generally accepted that mAChR subtypes are distributed as follows; in the glandular tissue the most prominent subtypes are the M_1 and M_3 (180), abundant expression of M_2 mAChR is observed in the heart (181) but also in the smooth muscle together with M_3 mAChR (182). The M_5 subtype has very restricted expression profile and can be found in cerebral blood vessels (178, 183).

Considering the wide distribution of mAChRs it is not surprising that these receptors mediate many important physiological functions. Among these can be mentioned reduction of heart rate, regulation of smooth muscle contractility and glandular secretion (184). The mAChRs are also involved in controlling various functions in the CNS such as cognitive, behavioral, motor and autonomic processes (178, 185, 186). Notably, many diseases of the CNS have been associated with changes in mAChR expression of which the best known are Alzheimer's disease, Parkinson's disease and schizophrenia (187).

1.4.1 mAChRs as targets for natural defense and attack

There are several compounds in the Nature which bind to mAChRs and either stimulate or inhibit the function of these receptors. These natural compounds can be found both from plants and from animals and have probably evolved to be part of the natural defense against predators.

Muscarine, a highly toxic alkaloid, was isolated in 1869 from the mushroom *Amanita muscaria*. Thereafter it has been found also from other mushrooms such as from *Inocybe* and

Clitocybe species where it is more abundant. Muscarine mimics the functions of acetylcholine by binding to and activating the mAChRs. If ingested, muscarine can cause poisoning characterized by bradycardia, bronchoconstriction and convulsions (188). This is due to muscarine binding to mAChR subtypes present in the heart and smooth muscle resulting in prolonged receptor activation. Cardiac arrest and respiratory failure have been reported in severe cases (189). The effects of muscarine can be reversed by mAChRs antagonists.

Natural antagonists of mAChRs include atropine, scopolamine and toxins isolated from snakes. Atropine is an alkaloid extracted from a perennial herbaceous plant, *Atropa belladonna*. This prototypic antimuscarinic drug acts by blocking the action of mAChR agonists in a competitive manner resulting in increased heart rate (190) and dilation of pupils (191). Scopolamine, an atropine-like alkaloid, was extracted from the same family of plants (nightshade family) as atropine in 1880 by Albert Ladenburg. It has a wider effect on the nervous system compared to atropine due to its ability to cross blood-brain barrier. Scopolamine has been used to dilate pupils, as a sedative and to prevent motion sickness. Side-effects of scopolamine include tachycardia and disorientation.

Toxins from snakes include both enzymes and non-enzymatic peptides. Snake venoms are known to be a rich source of phopholipase A_2, an enzyme that hydrolyses phospholipids into fatty acids and to other lipophilic substances, but only few of them show neurotoxic properties by binding to receptors on nerve and muscle membranes (192, 193). The muscarinic inhibitor from the Malayan spitting cobra is a phospholipase A_2 which was found to bind to the mAChRs and to block their actions (194). Regarding the non-enzymatic mAChRs blockers, MTs were discovered from green and black mambas at the end of the 1980's (195). Among these peptides can be found the most specific and selective ligands for the mAChRs.

1.5 Animal toxins

Toxins are substances produced by living cells or venomous organisms such as animals. They can be for example small molecules and proteins which are capable of interacting with biological macromolecules including receptors at the plasma membrane and enzymes. The effects of toxins can vary from minor to acute effects which in severe cases can lead to death.

1.5.1 Venomous animals

Venomous animals are widely spread in the animal kingdom comprising chordates (reptiles, fishes, amphibians, mammals), echinoderms (starfishes, sea urchins), molluscs (cone snails, octopi), annelids (leeches), nemertines, arthropods (arachnids, insects, myriapods) and cnidarians (sea anemones, jellyfish, corals) (196). Venoms of these animals have evolved to

immobilize, paralyze, kill and digest prey, but venoms can also be used for defense. The venom is stored in a gland from which it is "injected" to the prey through a specialized envenomation apparatus which can be a sting or a hollow fang (tooth).

The venom components of animals are well-selected by the Nature and targeted to interfere with vital physiological processes of the victim. Venoms comprise a unique mixture of peptides, proteins, carbohydrates, lipids and other small molecules such as alkaloids. The wide variety of different peptides from the venom has been proven to function as effective biological weapons. In many cases these peptides act on the nervous system and perturb neurotransmission but some are also able to disrupt host cell membranes, behave as antibacterials and exert cytolytic or hemolytic actions (197). In addition to the small peptides, several enzymes are found in the venoms such as phospholipases, peptidases and sphingomyelinases (198). Upon envenomation, these multiple peptides and enzymes work in cooperation to produce the desired effects.

The modern era of toxinology began in 1960's with the aim to understand the mechanism of action of lethal venom components and to create antivenoms for the envenomation victims (199, 200). Soon it became clear that venoms also contain non-lethal components that may be useful as research tools, as pharmaceutical lead compounds and as insecticides (201-206). A good example of the therapeutic potential of animal toxins is a conopeptide ω-MVIIA from the venom of the cone snail *Conus magus* (207). Its synthetic version, ziconotide, is used as the last line treatment for chronic pain in patients refractory to morphine and alternative treatments (207-209).

1.5.2 Snakes

Snakes are carnivorous reptiles which can be found on almost every continent. Currently there are over 20 different families of snakes which comprise about 500 genera and over 3000 different species. Of these ~ 20 % are considered venomous and include elapids, viperids, colubrids and atractaspidids. Snakes have always fascinated humans. They appear in many creation stories and are seen as symbols of fertility. In some cultures snakes are even considered immortal because of their ability to slough their skin. However, fascination and fear often go together.

The fear for snakes is an innate reaction in humans. Envenomation resulting from snake bite causes severe threats to human health leading in many cases to physical disability and even death. Mambas, which are of particular interest in this thesis, are one of the most venomous snakes and the mortality of envenomation reaches almost 100 % if proper therapy is not available. In a case report, a 31-year old man was bitten by a black mamba and developed

life-threatening symptomatology within 30 minutes (210). The man was rescued thanks to mechanical ventilation, myorelaxation and effective antivenin therapy.

Snake venoms can be considered as cocktails of peptides and proteins targeting primarily the peripheral nervous system and the neuromuscular junction of skeletal muscles. The protein components can be divided into different families based on their structure and function; to three-finger toxins, protease inhibitors, lectins, phospholipases, serine proteinases and metalloproteinases (211). The proteins within one family share remarkable structural similarity although in some cases their pharmacological actions can be quite different. For example some act on cell membrane proteins while the others inhibit the functions of certain enzymes.

α-bungarotoxin (αBTx), the first three-finger toxin isolated from the venom of the Taiwanese many-banded krait, *Bungarus multicinctus,* approximately fifty years ago, was found to bind with high affinity to the nicotinic acetylcholine receptors (nAChRs) and to block neurotransmission (212). After its discovery, αBTx has had important applications in biological research. This toxin, being selective for the α7 nAChRs, greatly facilitated the isolation and characterization of nAChRs (213). In addition to αBTx, several other snake peptides have been proven to be biologically valuable. Good examples are the bradykinin-potentiating peptides from the venom of *Bothrops jararaca* (venomous pit viper). Their molecular structures were used as a base for the design of a compound inhibiting the function of angiotensin-I converting enzyme (214, 215). This compound, named captopril, is currently used for the treatment of renovascular hypertension (215, 216).

1.5.3 Three-finger toxins

The three-finger toxin (TFT) family comprises one of the most abundant and well characterized families of snake venom peptides. These toxins are 60-74 amino acids long non-enzymatic peptides found in the venoms of elapids (cobras, kraits and mambas), hydrophiids (sea snakes) and colubrids (211, 217- 219). All TFTs are structurally highly conserved. They contain four or five disulfide bonds which direct the folding of these peptides into structures having three β-stranded loops protruding from a central core (Figure 8). The three loops projecting from the core resemble the three fingers of a hand thus explaining the name of the toxin family. However, the three-finger fold is not restricted solely to snake venom peptides as several non-venomous proteins and peptides have been shown to possess a similar folding pattern. Examples of these non-venomous peptides include the complement regulatory protein CD59 (220) and the Ly-6 alloantigen family members of the immune system (221) which includes lynx1. Lynx1 is a toxin-like peptide which is tethered to the plasma membrane through a GPI-tail (222). It was found to be present in the same cellular compartments as nAChRs where it altered the function of nAChRs (222, 223).

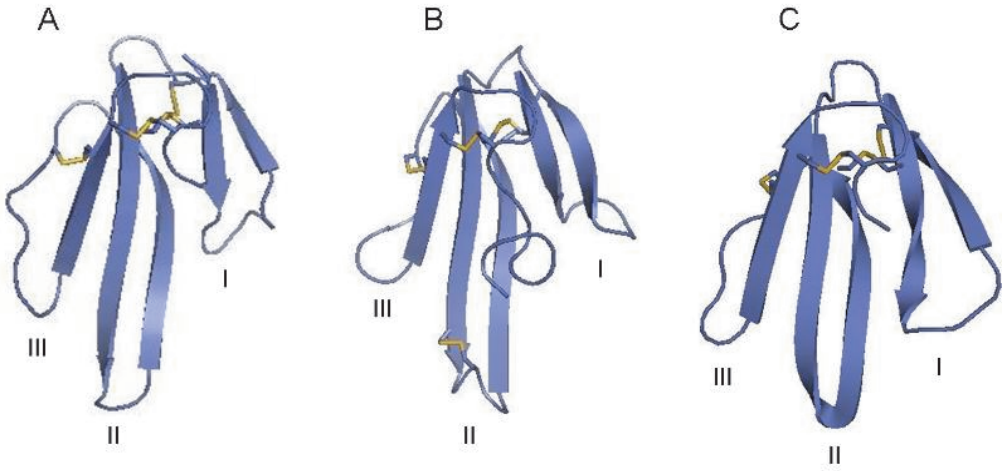

Figure 8. Three dimensional structures of three-finger toxins. A. Muscarinic Toxin 7 from the Eastern green mamba (MT7; pdb:2vlw), B. α-bungarotoxin from the Taiwanese many-banded krait (pdb; 1kfh) and C. Erabutoxin from broad-banded blue sea krait (pdb: 1qkd). The β-strands are presented as blue arrows and the disulfide bridges in yellow. Loop structures are presented as roman numbers I-III. The structures were drawn by the PyMOL Molecular Graphics System.

Regardless of their structural similarity, the three-finger toxins differ from each other in their pharmacological and biological properties. Most of the three-finger toxins identified interact with ligand-gated ion channels, in particular with the nAChRs (213). Additional TFT targets include voltage-gated ion channels (224, 225), coagulating factors (226), phospholipids (227), integrin receptors (228), acetylcholinesterases (229) and GPCRs (230-233). Consequently the three-finger fold appears to be a structural motif which allows a wide variety of unrelated but specific functions. Mamba venom seems to be a rich source of three-finger toxins. Most of the toxins currently found have been isolated from the black (*Dendroaspis polylepis*) and Eastern green mambas (*Dendroaspis angusticeps)* (224, 230-238); only a few are from Western green mamba and Jameson's mamba (195, 236, 239, 240). Among the three-finger toxins are toxins which target GPCRs including MTs and two recently identified novel toxins, ρ-Da1a and ρ-Da1b (230- 233).

1.5.3.1 Muscarinic toxins

Muscarinic toxins (MTs) are 65-66 amino acid long peptides isolated from both Eastern green mamba (*D. angusticeps*) and black mamba (*D. polylepis*) venoms. The first MTs (MT1 and MT2) to be isolated over 20 years ago from the venom of Eastern green mamba, were characterized by their ability to inhibit the binding of a nonselective muscarinic antagonist, quinuclidinyl benzilate (QNB), to muscarinic acetylcholine receptors (241, 242). Later on several other MTs were found from the Eastern green mamba (MT1-7) and black mamba (MTα, β and γ) that interfered with ligand binding to mAChRs (195). The venom of Western

green mamba (*D. viridis*) also contains toxins similar to MTs but these have not been characterized in detail (195, 240).

Two main research groups were responsible for isolating, characterizing and naming the MTs. Dr. Karlsson's group named the toxins according to the order by which they were discovered (243). According to this nomenclature MT1 and MT2 were the first toxins to be isolated from the Eastern green mamba whereas MTα and MTβ were the first toxins isolated from the black mamba and so on. Dr. Potter's group, instead, named the toxins based on the receptor for which they have the highest affinity (244). For example, m1-toxin shows the highest affinity for the M_1 mAChR whereas m4-toxin was found to be most active on the M_4 mAChR. Consequently, m1-toxin and MT7 are identical toxins. The same applies for m4-toxin and MT3. In the thesis, muscarinic toxins will be named according to Dr. Karlsson's group.

Muscarinic toxins are highly similar to each other at their primary sequence level (Figure 9). A closer inspection of toxin sequences reveals some highly conserved sequence signatures such as i) the eight cysteine residues involved in the formation of four disulfide bonds, ii) the LeuThrCysVal (LTCV) sequence present in the N-terminus, iii) the ThrAspLysCysAsnX (TDKCNX) sequence in the C-terminus, iv) GlyGlnAns(Leu/Val)CysPheLys (GQN(L/V)CFK) sequence connecting loops I and II and v) GlyCys(Ala/Val)AlaThrCysPro (GC(A/V)ATCP) sequence connecting loops II and III. Besides the toxins presented in Figure 9, two additional toxins, MT6 and MTγ, were isolated in the beginning of the 1990's (243, 245) but their amino acid sequences have not been determined. However, based on the pharmacological profile, MT6 is considered to be an isotoxin of MT3 (243). Three additional isotoxins of MT7 have also been found. These have single or a few amino acid substitutions in their primary sequences as compared to MT7, but these substitutions do not seem to affect the pharmacological profile of the toxins (246).

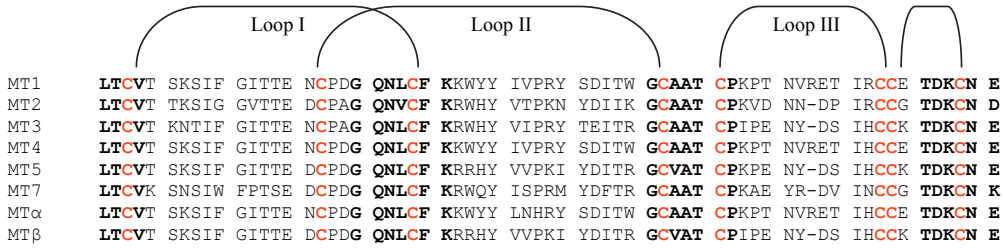

Figure 9. Amino acid sequence of the muscarinic toxins. The specific sequence signatures are shown in boldface and conserved cysteine residues are presented in red. Disulfide bridges are indicated by connecting lines.

1.5.3.1.1 Subtype selectivity and affinity for mAChRs

Conventional mAChR ligands usually lack specificity and selectivity for one particular receptor subtype. For example pirenzepine, a commonly used M_1 mAChR antagonist, shows only 2-43 fold higher affinity for the M_1 mAChRs as compared to the other mAChR subtypes (247). On the contrary to the conventional ligands, muscarinic toxins have been shown to be exceptional in their selectivity for a certain receptor subtype which offers remarkable advantages for the basic biological research.

The first indication regarding the subtype selectivity of MTs arose from MT1 and MT2. These MTs could not totally inhibit the specific binding of the tritium (^3H) labeled muscarinic antagonist QNB to rat and bovine cerebral cortex membranes (241). As QNB displays similar binding affinities for all the mAChR subtypes, it was suggested that these two MTs might bind to mAChRs in a subtype-selective manner (241). Further studies confirmed the subtype selectivity of MT1 and MT2 for the M_1 and M_4 mAChRs (Table 3, 248-250). Similar selectivity pattern was suggested for MT4 which differs from MT1 by a single amino acid at position 57 (His for MT4 and Arg for MT1) (Table 3, 251). However, to my knowledge the binding of MT4 to mAChRs has not been confirmed with cloned mAChRs. In addition to toxins above, also MT5 has been reported to target the M_1 and M_4 receptors (Table 3, 249).

MT3, also known as m4-toxin, binds to M_4 mAChR with high affinity ($pK_i = 8.7 \pm 0.06$) (Table 3, 252). A lower affinity (about 40-fold) has been measured for the M_1 mAChRs whereas the other mAChRs, M_2, M_3 and M_5, do not show detectable binding of MT3 (Table 3, 252, 253). The high affinity and selective binding of MT3 to the M_4 mAChRs has later been confirmed in functional assays using different cell lines and both recombinant and native receptors (254, 255). MT6, not yet sequenced, has been speculated to be an isotoxin of MT3 based on its amino acid composition and pharmacological profile (243, 249).

A toxin targeting the M_1 mAChR with remarkably high affinity and selectivity was found in the early 1990's by Dr. Potter's group and was named m1-toxin (244). Few years later Karlsson's group identified another toxin which bound selectively to the M_1 mAChR (256). The toxin was named MT7. Later these toxins were found to be identical despite initial errors in amino acid sequencing (246, 257). MT7 (m1-toxin) targets the M_1 mAChRs with picomolar (pM) affinity showing no detectable binding to the other mAChR subtypes (Table 3, 244, 258-260).

Three toxins resembling MTs from the Eastern green mamba, MTα, MTβ and MTγ, were isolated from the black mamba in the mid 1990's (245). MTα was initially reported to have high affinities for all the mAChRs (245). This has not, however, been confirmed. MTβ was also reported to bind to all mAChRs, but with lower affinities than MTα (245). Yet, a recent

study employing synthetic MTβ suggests that this toxin targets adrenoceptors and not mAChRs (233). MTγ has not been characterized and thus its selectivity pattern remains to be established.

Table 3. Subtype selectivity of muscarinic toxins from the Eastern green mamba for mAChR subtypes.

Toxin	Subtype selectivity	Reference
MT1	$M_1 > M_4 >> M_2, M_3, M_5$	261, 262
MT2	$M_1 > M_4 >> M_2, M_3, M_5$	261, 262
MT3	$M_4 >> M_1 >> M_2, M_3, M_5$	252, 253, 255
MT4	$M_1 > M_4 >> M_2, M_3, M_5$	251
MT5	$M_1 > M_4 >> M_2, M_3, M_5$	249
MT6	$M_4 >> M_1 > M_2, M_3, M_5$	249
MT7	M_1, no detectable binding to M_2, M_3, M_4, M_5	79, 258, 263

Individual affinity values can be found from the references.

Generally MTs are considered as antagonists although MT1 and MT2 have shown also agonistic properties. First indications regarding their agonism were obtained from studies examining the effects of MT1 and MT2 on memory (240, 264). Both of them were shown to behave like the mAChR agonist oxotremorine in the passive learning avoidance task in rats, and this behavior was antagonized by scopolamine (240, 264). However, in a recent publication Dr. Servent and co-workers reported that high concentration of synthetic MT1 could not induce Ca^{2+} release in cells expressing cloned M_1 mAChRs (258). This creates doubts on its agonistic actions. Another rather peculiar observation regarding agonistic actions of MTs arises with MT2. This toxin was reported to increase intracellular Ca^{2+} levels in cell lines expressing M_1, M_3 and M_5 mAChRs (265) although no binding to M_3 and M_5 mAChRs has previously been observed (Table 3, 248, 250). During my doctoral studies I have also tried to activate M_1 mAChRs with venomous MT2 (obtained from Peptide Institute, Japan) but without success.

1.5.3.1.2 Determinants of subtype selectivity

The interaction between a particular receptor and toxin is a result of good molecular fit. The initial clues considering the factors determining the subtype selectivity of MTs were based on the toxin sequences. By comparing the amino acid sequences and their pharmacological profiles, particular regions and amino acids possibly involved in the binding and defining the subtype selectivity could be indicated. For example, the charged residues present in MT1 and MT7 sequences are differently distributed. This might explain the differences seen in their

subtype selectivity and be one reason for the high affinity interaction between MT7 and M_1 mAChR (258).

The interaction occurring between MT7 and M_1 mAChR has been extensively studied both at the toxin and receptor level. An initial step towards understanding this interaction was provided by Krajewski and co-workers in 2001 (266). As only seven of the 65 amino acids of MT7 are not conserved in the other MTs, two of these amino acids, Phe38 and Lys65, were chosen and mutated to corresponding residues in other toxins; Phe38 to Ile and Lys65 to Glu. Neither of these two residues was observed to be important for the selective binding to the M_1 mAChRs (266). A study conducted in 1993 by Max and co-workers demonstrated that MT7 makes highly stable and irreversible interactions with the M_1 mAChR (267). However, a similar phenomenon was not observed to occur with Phe38Ile-MT7 mutant which dissociated rapidly from the receptor (266). The altered binding characteristics of Phe38Ile-MT7 mutant suggested that Phe38 might contribute to the stability of the toxin-receptor complex (266). On the contrary to Phe38Ile, Lys65Glu-MT7 mutant bound irreversibly to ligand-free M_1 mAChR but unlike native MT7, this mutant did not slow the dissociation of [^{3}H]-NMS. Based on this it was suggested that the positively charged lysine at position 65 forms contacts with an outer loop of the M_1 mAChR. (266)

The tips of three loops have previously been found to be the main determinants for the high affinity interactions of TFTs with nAChRs (268, 269). Based on this observation, several residues located mainly at the tips of these three loops were selected and mutated to alanines in MT7 (270). The amino acids residing at the tip of loop II (Arg34, Met35 and Tyr36) were noticed to be of particular importance for the high affinity binding to ligand-free M_1 mAChR (270). Based on the three dimensional structures of MT2, Arg34 has previously been proposed to be in a favorable position to interact with the amino acids residing in the ligand binding cavity of the target receptor (271). Substitution of the highly conserved Arg34 with an alanine resulted in approximately 200-fold reduction in the affinity for the M_1 mAChR. The other two residues, Met35 and Tyr36, mutated to alanines produced 8- and 20-fold reductions in the binding affinity. (258, 270). Further studies have shown that replacing the whole loop II of MT7 with that of MT1 decreases the affinity of MT7 for the M_1 mAChR significantly (~ 400-fold decrease) thus indicating the importance of loop II for the high affinity interaction (272). Substituting MT7 loops I and III by those of MT1 does not show significant impacts on the affinity of MT7 (272) although some modifications at the loops I (Trp10 → Ala and Ser8 → Ala) and III (Tyr51 → Ala) have been observed to have weak effects on toxin's affinity for the M_1 mAChR (270).

The mAChR subtypes are closely homologous to each other in their TMs and therefore a common binding site for MTs has been proposed (79). The selective binding of MT7 to M_1

mAChR must then be a result of some specific interactions taking place for example in the more divergent receptor domains. To identify the larger domains responsible for the selective interaction of MT7 with the M_1 mAChR, chimeras of M_1 and M_3 mAChRs were constructed (79, 80). The exchange of M_1 mAChR ECL1 with that of M_3 mAChR did not significantly alter the affinity of MT7 for the M_1 mAChR. However, when the ECL2 were exchanged drastic changes were observed in the affinity for MT7 (79, 80). Simple introduction of the ECL2 of M_3 mAChR on to the M_1 mAChR reduced MT7 binding by three orders of magnitude whereas introduction of the ECL2 of M_1 mAChR on to the M_3 mAChR converted M_3 mAChR into a receptor able to interact with MT7 (80). The ECL3 was also found to take part in MT7 binding (79).

As many other GPCRs, the M_1 mAChR is able to form dimers (273). MT7 was observed to bind to a dimeric form of M_1 mAChR and to promote its stability probably by inducing conformational changes within the receptor dimers (274). The stabilizing effect of MT7 on dimer formation was detected based on its ability to protect M_1 mAChRs from the dissociating effect of detergent, n-dodecyl-β-d-maltoside (274). Modeling dimeric M_1 mAChRs in complex with MT7 has provided further information regarding the high affinity interaction. The loops of MT7 interacted with the different protomers of the M_1 mAChR dimer; loops II and III interacted with protomer A whereas loop I made contacts with protomer B (80). The model also demonstrated that MT7 was flanked by the ECL2 of each M_1 mAChR protomer. This finding confirmed the crucial role of this loop structure in the interaction between M_1 mAChR and MT7.

At the receptor level several amino acids have been noticed to be important for the selective interaction occurring between M_1 mAChR and MT7. In the proximal part of the ECL2 Glu170, Arg171, Leu174 and Tyr179 have been identified to be crucial for the selective interaction with MT7 (79, 80). Other residues taking part in MT7 binding are Trp400 located at the TM7 (80) and Glu397 at the ECL3 (79, 275) although some conflicting results have been reported regarding the role of Glu397 in MT7 binding (79, 80, 275). It was suggested, based on the structural model of MT7-M_1 mAChR dimer, that the π-cation interaction between the conserved amino acids Tyr179/Trp400 of the M_1 mAChR and Arg34 of MT7 is one of the major determinants in binding whereas the electrostatic interaction between Glu170 of M_1 mAChR and Arg52 of MT7 is crucial for the specificity of MT7/M_1 mAChR interaction (80).

1.5.3.1.3 Binding mode of muscarinic toxins

Ever since the discovery of MTs as high affinity ligands for mAChRs, their mode of interaction has been under intensive research. The experiments performed have focused on determining whether the antagonism produced by MTs is competitive or non-competitive and

how stable are the toxin-receptor complexes formed. Regarding antagonism, muscarinic toxins have shown both competitive and non-competitive antagonistic effects on the mAChRs.

The binding mode of MT1 and MT2 is currently relatively unclear. For both toxins competitive interactions with the M_1 mAChR have been demonstrated (261). The competitive binding of MT1 has been later supported by radiolabeled MT1 whose binding to porcine brain was inhibited by pirenzepine (276) and by the lack of effect of MT1 on the dissociation kinetics of [^{3}H]-N-methylscopolamine (NMS) (258). However, other results have suggested an allosteric interaction for MT1 and M_1 mAChR as MT1 binding was not affected by 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) which is an irreversible orthosteric alkylating agent (240). On the contrary to MT1 and MT2, the binding of MT3 to M_4 mAChR has been more straightforward to determine. MT3 was shown to interact with the M_4 mAChR in a competitive manner (253-255, 277).

The binding mode of MT7 has been most intensively studied because of its high affinity and selectivity for the M_1 mAChR. MT7 binds to M_1 mAChRs in a non-competitive manner as judged from its disability to completely inhibit the binding of orthosteric ligands to the receptor (258, 259, 263, 266). In addition, if orthosteric ligands are added prior to MT7, the toxin appears to trap the ligands in the receptor (263). MT7 markedly decreases the rate of atropine-induced dissociation of [^{3}H]-NMS from the M_1 mAChRs (263, 267). On the other hand, MT7 accelerates the dissociation of [^{3}H]-ACh (259). Both of these observations on orthosteric ligand affinity indicated that MT7 acts as an allosteric modulator of M_1 mAChR. Additional evidence for the allosteric properties of MT7 came from studies where the interaction of MT7 with free and NMS-occupied M_1 mAChRs was examined (260). The results highlighted a strong negative co-operativity between MT7 and NMS which is characteristic for ligands that bind to an allosteric site and reduce the affinity of orthosteric ligands (65). These results thus provided further evidence that MT7 interacts with M_1 mAChR through an allosteric site.

Toxins can form either reversible or irreversible/pseudo-irreversible interactions with their target receptors. In reversible binding the toxin-receptor complexes are rapidly formed and deformed whereas in irreversible/pseudo-irreversible binding the toxin and receptor form rather stable interactions which are not easily broken. MT7 forms highly stable and irreversible interactions with the M_1 mAChR. This has been revealed by the inability of [^{3}H]-NMS or [^{3}H]-pirenzepine to interact with the M_1 mAChR after receptors have been preincubated with MT7 (263, 266, 267). MT1 and MT2 have also been reported to interact irreversibly with M_1 and M_4 mAChRs (262) although some studies have suggested that MT1

binding is reversible (258, 278). For the MT3 reversible binding has been confirmed (254, 255).

1.5.3.2 Three-finger toxins acting on adrenoceptors

Among the three-finger toxins, there are also toxins that are able to interact with adrenoceptors and block their functions. In a doctoral thesis from 1996, the author briefly describes a moderate affinity interaction of MT3 with α_1- and α_2-ARs (249). A few years later Dr. Harvey and co-workers conducted a study on the functional effects of MT1 and MT2 on different tissue preparations (262). This study revealed that both MT1 and MT2 could reversibly inhibit the binding of [^{3}H]-prazosin (α_1-AR antagonist) to rat cerebral cortex and vas deferens membranes (262). Of the two toxins MT1 appeared to be more potent on the α_1-ARs than MT2.

During the course of this study, the Eastern green mamba venom was extensively screened in hope to discover new toxins acting on aminergic-GPCRs. Two novel three-finger peptides isolated, ρ-Da1a (initially named AdTx1) and ρ-Da1b, were both active on α-ARs and displayed high sequence similarity to MTs (230, 231). The amino acid sequence of ρ-Da1a is very similar to MTβ (97 % identity) (230, 245) whereas ρ-Da1b shows some structural similarity to MTα (77 %) and MT3 (76 %) (231). ρ-Da1a has subnanomolar affinity for the α_{1A}-AR and approximately 1000 times lower affinity for the other α_1-AR subtypes (230). The activity of ρ-Da1a was also tested on the α_2-ARs, β-ARs and mAChRs. ρ-Da1a inhibited α_2-ARs at high micromolar concentrations while β-ARs and mAChRs were unaffected by the toxin (230). ρ-Da1b was found to target the α_2-ARs (231). This toxin shows slight selectivity for the α_{2A}-AR subtype over the other α_2-ARs (ninefold compared with α_{2C}-AR and fivefold compared with α_{2B}-AR).

Additional three-finger toxins, MTβ and CM-3, acting on ARs were discovered from black mamba venom over fifteen years ago (245, 279). Initially MTβ was reported to bind non-selectively to mAChRs (245) while no biological function was determined for CM-3 (279). Both MTβ and CM-3 show high sequence identity to MTs and ρ-Da1a. For this reason synthetic MTβ and CM-3 were pharmacologically re-characterized by Blancet and co-workers (233). Their study revealed that synthetic MTβ and CM-3 have very similar pharmacological profiles on the three α_1-AR subtypes and on α_{2C}-AR. Both of them are very potent on all α-ARs tested; highest affinity was measured for the α_{1A}-AR. The activities of MTβ and CM-3 resemble that of ρ-Da1a but they interact more potently with the α_{1B}- and α_{1D}-ARs as ρ-Da1a does (230, 233).

2 AIMS OF THE STUDY

Adrenoceptors have proved to be important mediators of a number of physiological responses. Impairments in the function of these receptors can lead to severe diseases such as to cardiovascular diseases and BPH. The most well-known drugs targeting ARs are the beta-antagonists which have been used to treat angina pectoris for over 40 years. However, many drugs targeting ARs lack specificity and selectivity which prevent their use as therapeutics. The aim of this study was to find new receptor ligands which would show selectivity among the AR subtypes. Muscarinic toxins from mamba snakes have been shown to bind to mAChRs with high selectivity. Thus the interaction of these toxins with the ARs was studied and characterized.

Specific aims were as follows

- The initial studies have indicated that MTs from the mambas interact with mAChR with rather high affinity and selectivity. However, few studies have shown that MTs are able to interact also with the α_1- and α_2-ARs. In publications I and III, the binding of four different MTs to the ARs was evaluated and their binding to the mAChRs reassessed. (I, III)

- To characterize further MTα binding to the α_{2B}-AR by analyzing the effect of MTα on various parameters of agonist and antagonist binding. Additionally, MTα binding sites on the α_{2B}-AR were mapped with the help of chimeric α_2-ARs. (II)

- Several toxins, such as α-BTx have been anchored to the plasma membrane with the help of a GPI-tail. Thus it was evaluated if such GPI-anchoring could be applied to MTs targeting the mAChRs and ARs. (IV)

3 EXPERIMENTAL PROCEDURES

This section introduces the methods used in the publications (I-IV). For more precise information on the methods and materials used the reader is referred to individual studies.

3.1 Receptor expression

The receptors were recombinantly expressed in *Spodoptera frugiperda* insect cells (Sf9) with the help of the Bac-to-Bac® expression vector system (Invitrogen, Paisley, UK). The generation of recombinant baculoviruses is based on the site-specific transposition where the gene to be expressed is first cloned into a plasmid transfer vector, pFastBac1 in this study. This recombinant plasmid is then transformed into competent DH10Bac *Escherichia coli* cells containing the bacmid DNA. The transposition process is enabled by proteins provided by a helper plasmid. Bacterial colonies containing recombinant bacmid DNA are identified by disruption of the lacZα gene. The recombinant bacmid DNA is then used to transfect Sf9 insect cells. The viral stocks harvested from the transfected insect cells are used to infect fresh insect cells to induce recombinant receptor expression. (280).

In the Bac-to-Bac® system, the gene of interest replaces the polyhedrin gene and becomes under the control of polyhedrin promoter. However, the polyhedrin promoter is expressed late in the infection cycle when the lytic baculoviruses are already killing the host cells. To induce receptor expression earlier in the infection cycle, the receptor genes were cloned behind the Rous sarcoma virus promoter (RSV-promoter) in some cases. The RSV-promoter is a mammalian virus promoter that appears to work well in the Sf9 insect cells infected with recombinant baculoviruses (281).

3.2 Chimeric receptor constructs and mutagenesis

Chimeric receptors can be used to study the major domains involved in ligand binding. Six different chimeric α_2-ARs were constructed using polymerase chain reaction (PCR) based mutagenesis. Oligonucleotide primers used in the publication II are presented in Table 4. Additional restriction sites to either α_{2A}-AR or α_{2B}-AR sequence were introduced by PCR to facilitate subcloning. In order to make AB-I chimera a BglII restriction site was introduced into the α_{2B}-AR sequence (primer BBGCS). AB-II chimera was generated by introducing an ApaI restriction site into α_{2A}-AR sequence (primer AAPNCS). For AB-III it was necessary to introduce a StuI restriction site into α_{2A}-AR sequence (primer ASTNCS) whereas an AccI restriction sites at equivalent positions in both α_{2A}-AR and α_{2B}-AR were used to generate the AB-IV chimera (Figure 10A). An Eco47III restriction site is located at the same position in all

three α_2-ARs and this was utilized to generate the BA and BC constructs (Figure 10B). In addition to the chimeric α_2-AR constructs, two mutant α_{2B}-AR variants were made. Amino acids Asp153 (α_{2B}-Asp153Ala) and Gnl154 (α_{2B}-Gln154Ala) were mutated to alanines using standard PCR techniques.

Table 4. A list of primers used for mutagenesis in publication II.

Primer name	Primer sequence
BBGCS (forward) (=AB-I)	5'-GC GAG ATC TAC CTG GCG CTC GAC GTG CTC-3' Bgl II
AAPNCS (reverse) (= AB-II)	5'-CC GGG GCC CTT CTT CTC GAT GGA GAT GAG CGG CG-3' Apa I
ASTNCS (reverse) (=AB-III)	5'-A CCA GGC CTG GTC GTT GAT CTC GCA GCG CGG CT C-3' Stu I
BDANCS (reverse) (=α_{2B}-Asp153Ala)	5'- TG GGG GCC CTG GGC GCC CTT GTA GAT GAG GGG C -3'
BQANCS (reverse) (=α_{2B}-Gln154Ala)	5'- TG GGG GCC CGC GTC GCC CTT GTA GAT GAG GGG -3'

Nucleotides encoding an artificial restriction enzyme recognition site are highlighted in red. Underlined red nucleotides are those changed compared to the wild type nucleotide sequence. Nucleotides encoding a single amino acid mutation are highlighted in green. Template for the primers was either α_{2A}-AR (AAPNCS and ASTNCS) or α_{2B}-AR (BBGCS, BDANCS and BQANCS).

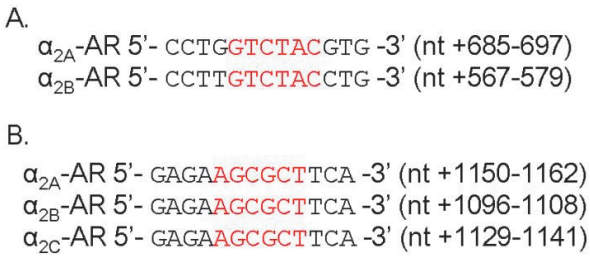

Figure 10. Stretches of α_2-AR sequences showing the AccI and Eco47III restriction sites. A. AccI restriction sites at equivalent positions in both α_{2A}-AR and α_{2B}-AR. B. Eco47III (AfeI) sites in the α_2-ARs. Restriction enzyme recognition sites are highlighted in red. Numbers in brackets indicate the position of sequence stretches in the α_2-ARs.

3.3 Radioligand binding

Radioligand binding assays are commonly used to determine receptor-ligand interactions. The binding reaction between a receptor and a radiolabelled ligand can be described by the following simple, reversible and bimolecular reaction, where k_{on} is the association rate of a ligand (L) for the receptor (R) and k_{off} is the dissociation rate of a ligand from the receptor (Equation 1). (282)

$$R + L \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} RL \qquad \text{Equation 1}$$

In the present studies (I-IV) several different radioligand binding assays were performed to characterize toxin binding to the ARs: saturation binding experiments, radioligand displacement experiments, kinetic titrations and experiments designed to reveal the reversibility of toxin binding to the receptors. Radioligands used in publications I-IV are presented in Figure 11.

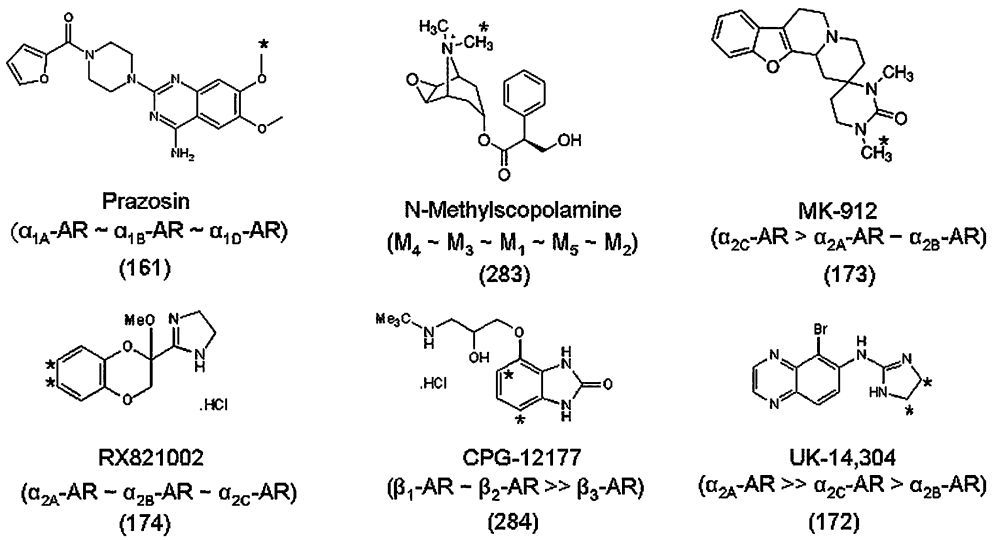

Figure 11. ^{3}H-labeled radioligands used in the publications (I-IV). Binding preferences of the compounds are shown in brackets. References for the binding preferences are shown below brackets. Of the compounds used UK-14,304 is an agonist and the others are antagonists. * Indicates the position of the radioactive isotope.

3.3.1 Saturation binding experiments

The affinity of a radiolabeled ligand for a receptor can be determined in saturation binding experiments. In these experiments receptors are incubated with different concentrations of radioligand until equilibrium state is reached and total binding obtained. Non-specific binding must be determined and subtracted from the total binding. The non-specific binding is usually determined by using a high concentration of unlabelled ligand which will occupy receptor binding sites studied. After plotting the specific binding vs. radioligand concentrations, two different parameters can be obtained; K_d and B_{max} values. K_d is the equilibrium dissociation constant of the radioligand and describes the strength of interaction of the ligand with receptor whereas B_{max} value describes the maximum density of receptors present in the assay and is often presented as mol/mg of protein. (282)

The following radioligands were used to obtain K_d and B_{max} values of the adrenoceptors and mAChRs; [^{3}H]-prazosin for α_1-ARs, [^{3}H]-MK-912 and [^{3}H]-RX821002 for α_2-ARs, [^{3}H]-UK-14,304 for α_{2B}-AR, [^{3}H]-CGP-12177 for β-ARs and [^{3}H]-NMS for mAChRs. The unlabelled ligands used to determine non-specific binding were phentolamine for α_1- and α_2-ARs, propranolol for β-ARs and atropine for mAChRs.

3.3.2 Radioligand displacement experiments

In the radioligand displacement experiments, binding of the radioligand to the receptor is measured in the presence of various concentrations of unlabelled ligand (in these studies the toxins used). Increasing the concentration of unlabelled ligand results in increased displacement of the radioligand. The usual parameter obtained from radioligand displacement experiments is the IC_{50} value which represents the concentration of an inhibitor decreasing the binding of the radioligand to the receptor by 50%. For truly competitive ligands, the IC_{50} values can be converted to K_i values which describe the affinity of the unlabelled ligand taking into account the concentration of the radioligand and its affinity for the receptor. However, when studying the toxin molecules, we found that the IC_{50} values were unaffected by the radioligand concentrations. With MTα the radioligand used ([^{3}H]-RX821002, [^{3}H]-UK-14,304, [^{3}H]-MK-912) neither affected the IC_{50} values. For this reason we preferred to list IC_{50} values instead of K_i values. (282)

3.3.3 Kinetic experiments

Kinetic binding experiments are used to measure radioligand binding at various times to determine its dissociation (k_{off}) and association (k_{on}) rates. Also the effect of unlabelled ligands on the radioligand binding kinetics can be determined by using these assays. In dissociation experiments receptors are first incubated with the radioligand until equilibrium is reached. Thereafter dissociation is started by adding a displacing concentration of an

unlabelled competitor. After dissociation has started, binding is measured over time to determine how quickly the ligand dissociates from the receptors. Association experiments are useful in determining how long it takes before equilibrium is reached in saturation binding experiments. In these assays the radioligand is added and specific binding is measured at various times. (282). Ligands binding to an allosteric site can affect the receptor in such a way that the association and/or dissociation rates of orthosteric ligands are altered. For MTs, such phenomenon has been readily shown with MT7 and the M_1 mAChR (259, 263, 266).

3.3.4 Reversibility of toxin binding

The longevity of receptor-toxin complexes can be determined by studying the reversibility of the interaction with the help of radioligands. In publication I we applied a method where cell homogenates were centrifuged and re-suspended up to four times. The recovery of receptor binding sites was almost 100%, both in control experiments and when preincubated with MTα (I).

3.4 Functional calcium mobilization assays

There are several methods which can be employed to study receptor activation and intracellular signaling cascades. One common method is the Ca^{2+} mobilization assays where increases in intracellular calcium levels are monitored with the help of fluorescently labeled compounds. In these studies the fura-2 probe was used. This probe is highly selective for Ca^{2+} and it is delivered into the cells as an acetoxymethyl ester conjugate (fura-2-AM). In cells fura-2-AM undergoes removal of the AM group and as a result of this fura-2 gets trapped into the cells. Upon Ca^{2+} binding fura-2 undergoes a spectrum shift on which the measurements are based on. In the absence of Ca^{2+} the wavelength of fura-2 for maximal excitation is 380 nm which is transferred to 340 nm upon Ca^{2+} binding. Receptors coupled to $G\alpha_{q/11}$ result in increases in intracellular Ca^{2+} levels. For receptors predominantly coupled to $G\alpha_{i/o}$ in Sf9 cells (α_{2A}-AR, α_{2C}-AR and M_4 mAChR), a chimeric G-protein $G_{11\alpha i5}$ ($G\alpha_{11}$:$G\alpha_i$ chimera) was co-expressed in the assays.

4 RESULTS AND DISCUSSION

Animal venoms are complex and diverse mixture of pharmacologically active peptides and proteins. The huge diversity of venomous peptides is the most apparent within small peptides such as conopeptides and conotoxins present in the venom of cone snails; their venom accounts > 50 000 different peptides (201). Snake venoms are another diverse source of pharmacologically highly active peptides which largely affect neurotransmission. Most of the peptides characterized from snake venoms target ligand-gated ion channels but some appear to interact specifically with GPCRs. Muscarinic toxins, TXTs from the black and Eastern green mambas, were previously identified as ligands acting on mAChRs (241, 244, 245, 251). However, the specificity of MTs for the mAChRs has not been extensively studied. This thesis work shows that muscarinic toxins are able to interact also with the α-ARs besides their previously identified mAChR targets. The toxin-receptor interactions occurring between MTs and α-ARs were found to occur with high affinity and in the case of MTα with high specificity.

The results described here present the main results from publications I-IV.

4.1 Adrenoceptor activity of muscarinic toxins (I, III)

Initially, muscarinic toxins were identified as toxins binding with high affinity and specificity to the mAChRs. An implication that these toxins might be active on ARs was first presented by Mikael Jolkkonen who reported in his thesis work that MT3 interacts with the α_1- and α_2-ARs with moderate affinity (pK_i between 6 and 7) (249). However, no additional data has been presented to support this. The specificity of MTs for the mAChRs was further challenged by Dr. Harvey and co-workers who noticed that MT1 and MT2 interfered with ligand binding to the α_1-ARs (262).

The AR activity search was initiated with venomous MTα which was a generous gift from Dr. E. Karlsson (I). Previously venomous MTα has been reported to bind to all mAChRs with a rather high affinity (pK_i 7.36-8.46) (245) but in our hands the toxin did not produce inhibitory effects on the M_3 mAChRs in functional calcium mobilization assay (I, Figure 12A). Instead, incubation of venomous MTα with the α_{2B}-AR resulted in total block of receptor responses (I, Figure 12B). Further characterization of the blocking effect of MTα on the α_{2B}-AR showed that agonist induced increases in intracellular calcium levels ($[Ca^{2+}]_i$) were concentration dependently inhibited by the toxin (I). 300 nM MTα was required to fully silence the responses (I).

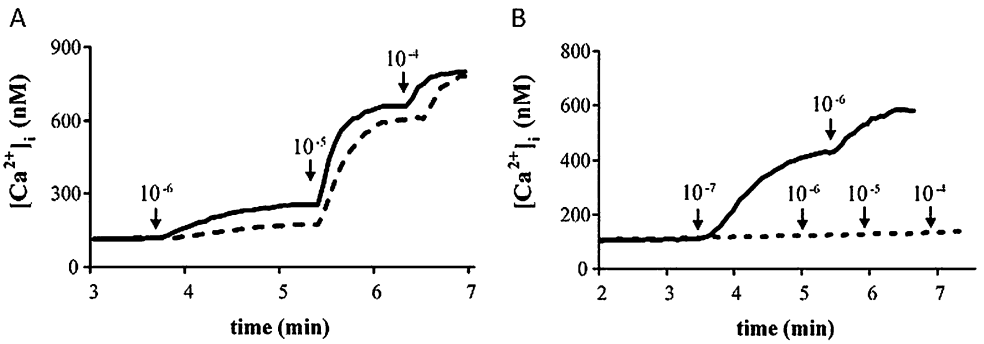

Figure 12. Blocking effect of venomous MTα. Suspensions of Sf9 cells were used to measure $[Ca^{2+}]_i$ fluctuations in response to receptor stimulation. Fluorescence traces obtained from intracellularly loaded fura-2 were converted to $[Ca^{2+}]_i$ and are shown as such. Two traces in each panel are superimposed. The continuous lines represent control condition and the dotted lines represent the presence of 1.8 μM venomous MTα. Numbers at arrows indicate molar additions of agonists. A. Cells expressing the human M_3 mAChR stimulated with carbachol. B. Cells expressing the human α_{2B}-AR stimulated with noradrenaline. Reprinted with permission from publication I.

The antagonistic potency of synthetic MTα was further screened with all members of the α-adrenoceptors and the mAChRs and with the β_1- and β_2-ARs (I, III). Results indicated that MTα interacts specifically and with high affinity with the α_{2B}-AR (IC_{50} = 2.5-3.5 nM depending on the radioligand used in the assay) leaving the other adrenoceptors and mAChR subtypes unaffected (Figure 13A and B, Table 6). The subtype diversity of ARs has put pressure on the development and synthesis of selective and specific ligands for these receptors. Regarding the ligands acting on the ARs, most are rather unselective and show binding even to other GPCRs. This is a significant disadvantage considering their role as therapeutic agents and experimental tools. However, some ligands have shown to be somewhat selective for a certain receptor subtype. Among these are the α_2-AR ligands JP-1302 and MK-912. Of these, JP-1302 shows about 100-fold selectivity for the α_{2C}-AR over the α_{2A}-AR and α_{2B}-AR subtypes (165) while MK-912 is about 4- and 13-fold more selective for the α_{2C}-AR compared with the α_{2A}-AR and α_{2B}-AR (167). Although subtype selective ligands have been developed and found for the other AR subtypes, the α_{2B}-AR has lacked them. This feature makes MTα unique as it is currently the only ligand targeting this AR subtype with high affinity and remarkable selectivity.

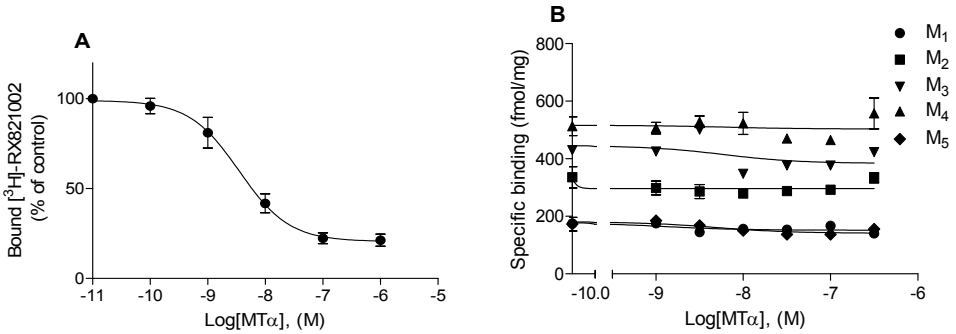

Figure 13. Inhibition profile of synthetic MTα on the α_{2B}-AR and mAChRs. A. MTα inhibiting specific [^{3}H]-RX821002 binding to the α_{2B}-AR. Data points are means ± SEM from three experiments. B. The effect of MTα on mAChR subtypes. [^{3}H]-NMS was used to label mAChRs. Data points are means ± SD from one experiment performed with triplicate samples. The figure is adapted and reprinted with permission from figures 4B and 7 from publication I.

Considering the lack of interaction between mAChRs and synthetic MTα, a similar phenomenon has been observed with the venom purified MTβ and its synthetic analog. The venom-purified MTβ has been reported to display micromolar affinity (pK_i 6.00-6.90) for the mAChRs (245). This is in contrast to the synthetic MTβ which shows highly potent interactions with the α-adrenoceptors and no or very weak binding to mAChRs (233). The pharmacological discrepancies detected between venom-purified and synthetic toxins might be a result of contamination in the venom fraction that might associate with their action on the mAChRs but also sequencing errors might have effects on the pharmacological outcome. Additionally the original publication presenting the micromolar interaction between mAChRs and MTα has no binding data presented to support the observation (245). Taking these into account, the activity of venomous MTα should be considered with some care as it has not been investigated thoroughly.

Encouraged by the results obtained with MTα, binding of MTs to ARs was further characterized with three additional toxins (III). Of the MTs chosen MT1 is known for its binding to the M_1 and M_4 mAChRs (258, 261), MT3 for its high affinity binding to the M_4 mAChRs (252, 255) and MT7 for its remarkable selectivity for the M_1 mAChR leaving the other mAChR subtypes unaffected (244, 256, 258-260). Table 6 presents the inhibition profiles of all the MTs used in the studies I and III. MT1 exhibited similar affinity for the α_{2B}-AR as MTα (III, Figure 14, Table 6). Low affinity binding was detected with the α_{2A}-AR and α_1-AR subtypes (Table 6). The mAChR ligand, [^{3}H]-NMS, was displaced by MT1 from the M_1 and M_4 mAChRs as previously described (251, 258, 261, Table 6).

Table 6. Inhibition profiles of four muscarinic toxins at different receptors.

	pIC_{50} ± SEM (n = 3-4)			
Receptor	**MT1**	**MT3**	**MT7**	**MTα**
α_{1A}	6.98 ± 0.17	8.86 ± 0.14	NI	NI
α_{1B}	<6.5 (48%)[a]	7.57 ± 0.22	NI	NI
α_{1D}	<6 (28%)[a]	8.13 ± 0.08	NI	NI
α_{2A}	<6.5 (47%)[a]	8.49 ± 0.06	NI	NI
α_{2B}	8.64 ± 0.10	<6.5 (39%)[a]	NI	8.62 ± 0.12
α_{2C}	NI	7.29 ± 0.13	NI	NI
β_1	NI	NI	NI	NI
β_2	NI	NI	NI	NI
M_1	6.85 ± 0.06	6.71 ± 0.14	9.36 ± 0.06	NI
M_4	6.54 ± 0.09	8.79 ± 0.06	NI	NI

The inhibitory potencies of different MTs were determined in radioligand displacement experiments. [a]% values in parentheses indicate inhibition obtained with 1 μM toxin because the inhibition curves did not reach saturation at concentrations used. NI = no inhibition. The threshold for inhibition was set to 25% at 1 μM concentration for MT1, MT3 and MTα and 0.3 μM for MT7 (III).

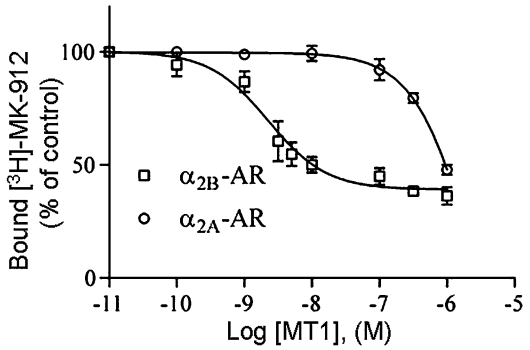

Figure 14. Inhibition profile of MT1 on the α_{2B}-AR and α_{2A}-AR. MT1 inhibiting specific [^{3}H]-MK-912 binding to the α_{2B}-AR and α_{2A}-AR. Data points are given as % of control binding and represent means ± SEM of three experiments each performed with triplicate samples. The data with the α_{2A}-AR serves as an example of inhibitory potencies when given as % inhibition in Table 6. Reprinted with permission from publication III.

Taking into account the selectivity profiles of MTα and MT1, small deviations in their amino acid sequences seem to have large effects on their affinity for adrenoceptor and mAChR subtypes. MT1 binds more tightly to its high affinity adrenoceptor targets (α_{2B}-AR and α_{1A}-AR) than to its previously identified target receptors M_1 and M_4 mAChRs (Table 6, 251, 258, 261). Similar affinities of MT1 and MTα for the α_{2B}-AR are not surprising taking into account the primary sequences of these two toxins; they differ from each other only by four amino

acids (Figure 15). Three of these amino acids are clustered at the tip of loop II, Ile31ValPro33 in MT1 and Leu31AsnHis33 in MTα, whereas the fourth amino acid difference, Arg57 in MT1 and His57 in MTα, is located adjacent to the third disulfide bridge making up the third loop. Residues adjacent to disulfide bridges are highly conserved among the MTs. It is more likely that Arg57/His57 ensures the correct disulfide bridge formation which is important for the right folding to take place than to have effects on receptor binding.

```
    1           10          20          30          40          50          60
MT1 LTCVT SKSIF GITTE NCPDG QNLCF KKWYY IVPRY SDITW GCAAT CPKPT NVRET IRCCE TDKCN E
MTα LTCVT SKSIF GITTE NCPDG QNLCF KKWYY LNHRY SDITW GCAAT CPKPT NVRET IHCCE TDKCN E
```

Figure 15. Amino acid sequences of MT1 and MTα. Amino acid differences of MT1 and MTα are highlighted in red.

The three amino acids at the tip of loop II are adjacent to Arg34. The residue at this particular position has been speculated to reach down to the ligand binding cavity of the orthosteric ligands and to interact with residues of the transmembrane helices allowing the amino acid residues near to Arg34/Lys34 to be in contact with the residues of the outer surfaces of the target receptor (271). The LeuAsnHis sequence of MTα is unique among MTs and thus it would be intriguing to name it as an α_{2B}-AR recognition motif. However, as the affinity of MT1 for the α_{2B}-AR is equal to that of MTα, it is not likely that LeuAsnHis sequence would alone determine the high affinity binding of MTα for the α_{2B}-AR but rather be a reason for the lack of binding to mAChRs. In this sense LeuAsnHis-motif would fulfill its function from the pharmacological point of view. If LeuAsnHis -motif in MTα is the reason for lack of binding to mAChRs, corresponding amino acids in MT1 might confer to mAChRs binding. The Tα/1L-GPI construct is based on MTα and MT1 sequences and has His33 of LeuAsnHis sequence of MTα replaced by Pro33 like in MT1 sequence (IV). This toxin construct inhibits the α_{2B}-AR induced receptor responses as the soluble MTα (see publication IV Figures 3B and 4) but it can also bind to the M_1 and M_4 mAChRs although with lower affinity as the wild type MT1 (258, 261). This indicates that Pro33 might have a role in mAChR binding. However, before firm conclusions can be made this issue needs to be tested with soluble toxins in detail as the membrane-anchored conformation can have effects on the pharmacological properties of the toxin.

MT3 has a large receptor repertoire (III). In addition to its action on the M_4 mAChRs (252, 253, 255, Table 6), MT3 shows high-affinity interactions with all three α_1-ARs and α_{2A}-AR (Figure 16, Table 6). Relatively high affinity was detected with the α_{2C}-AR (Figure 16B, Table 6). On the contrary to MTα and MT1, binding to the α_{2B}-AR was considerably low. This indicates that there are some structural differences in the α-ARs that preclude MT3 from

being a totally unselective ligand. The reason for these structural differences could arise from the extracellular loops of α-ARs which are not conserved among the α-AR subtypes and show much more divergence. Given that MT3 has a broad number of high affinity receptor targets and the fact that other MTs are able to show subtype selectivity, it could be possible to use MT3 as a template for the design of new and more subtype selective ligands for the receptor subtypes still lacking them. From the snake's point of view, the wide repertoire of receptor targets for MT3 might have evolved to strengthen the effect of other MTs as in the venom the toxins work in co-operation to produce the desired effects.

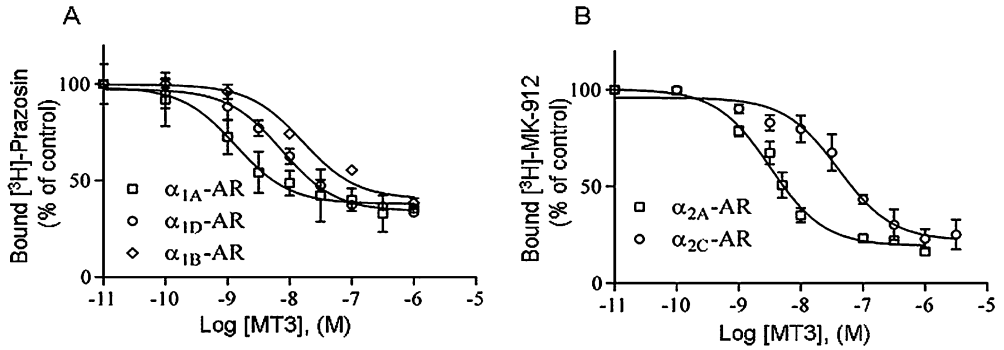

Figure 16. Inhibition profile of MT3 on the α-adrenoceptors. A. MT3 inhibiting specific [^{3}H]-prazosin binding to α_1-ARs. B. MT3 inhibiting specific [^{3}H]-MK-912 binding to α_2-ARs. Data points are means ± SEM of three experiments performed in triplicates. Reprinted with permission from publication III.

Several studies have shown that among mAChRs, MT7 displays remarkable selectivity for the M_1 mAChRs (244, 256, 258-260). MT7 has failed to show effects on the neuronal nAChRs as demonstrated by Max and co-workers (285) but its binding to other GPCRs has not been evaluated. Our studies demonstrate that MT7 does not bind to adrenoceptors confirming thus its high affinity and selective interaction with the M_1 mAChRs (III, Table 6). Considering the β-ARs, none of the toxins was able to inhibit [^{3}H]-CGP-12177 binding to β_1- and β_2-ARs although β-cardiotoxin, structurally related to MTs, was shown to bind to β-ARs (286). It might be that there are some structural differences between the β-ARs and α-ARs which prevent MT binding to the β-ARs. A reason for these structural differences could arise from the ECL2 which probably folds differently in β- and α-ARs due to presence of additional disulfide bridge in the ECL2 of β-ARs (15). Despite the fact that the MTs examined did not bind to β-ARs, it nevertheless appears that snake venom contains toxins that can target β-ARs, the highly important receptors involved in the maintenance of cardiovascular homeostasis.

4.2 The enigmatic receptor-toxin interactions (I, II, III)

Muscarinic toxins have been shown to behave both as competitive and non-competitive antagonists of receptor function. However, defining MTs into distinct pharmacological categories has in some cases been relatively difficult. For example MT1 and MT2 have been shown to function as competitive antagonists but even allosteric and agonistic interactions have been described (240, 258, 261, 276).

Muscarinic toxins used in the studies bound to their target receptors in a non-competitive manner with regard to classical orthosteric ligands. This was judged from the inability to displace all bound radioligand from the receptors (20-40 % of specific binding left at saturating concentrations of MTs) (I, II, III) and from the insurmountable inhibition of receptor responses (I, III). Similar inability to displace orthosteric ligands has previously been presented by MT7 and M_1 mAChRs (258, 259, 263, 266). In the case of MTα, further evidence in favor for non-competitive binding mode came from the similarity in IC_{50} values when different radioligands were used and from saturation binding experiments where the B_{max} values were suppressed as for non-competitive ligands in the presence of MTα (I, II). On the contrary to the B_{max} values, the affinities of radioligands (K_d values) for the α_{2B}-AR were not changed (I, II). However, it should be noted that MTα bound to the α_{2B}-AR in a weakly reversible manner (pseudo-irreversibly i.e dissociates slowly from the receptors) as extensive washing was needed to recover all binding sites at high toxin concentrations (I). This might have effects on the binding mode. Functional calcium mobilization assays additionally pointed that MTα, MT1 and MT3 bind rather slowly to their target receptors (I, III) which is different seen for example with MT7 which binds to the M_1 mAChR instantly after its addition (79, 259, 285).

The non-competitive binding mode of ligands has been in many cases ascribed to allosteric interactions between the ligand and receptor which has effects on the binding kinetics of orthosteric ligands (287). Allosteric modulators generally alter the affinity of orthosteric ligand for the receptor but changes can be detected also on the efficacy of the ligand or on both of these. The interaction between the M_1 mAChR and MT7 serves as a good example of an allosteric interaction (259, 263, 267). MT7 has an influence on the affinity of the orthosteric ligands for the M_1 mAChR; it slows down the dissociation rate of [^{3}H]-NMS (263, 266) and accelerates that of [^{3}H]-ACh (259). However, MT7 appears to be exceptional in this regard as another non-competitive TFT, ρ-Da1a, does not affect dissociation rates of orthosteric ligands from the α_{1A}-AR (230) indicating that the inhibitory actions are not governed through an allosteric site. To test whether or not the non-competitive binding of MTα was due to an allosteric interaction between MTα and the α_{2B}-AR, the effects of MTα on the orthosteric ligand binding kinetics were tested (I, II). The kinetic experiments were performed with three different radioligands of which two are antagonists ([^{3}H]-RX821002

and [^{3}H]-MK-912) and one an agonist ([^{3}H]-UK-14,304). MTα had no effects on the dissociation and association rates of radioligands for the α_{2B}-AR indicating that MTα does not exert its inhibitory actions through an allosteric mechanism (I, II). The α_{2B}-AR was also preincubated with the toxin before radioligands were added and dissociation started (I). Neither this had any effects on radioligand dissociation suggesting that MTα does not bind to ligand-occupied receptors. This feature of MTα is not in line with MT7 which can bind to ligand-occupied M_1 mAChRs and trap the orthosteric ligands to the receptor (263). The lack of allosterism was further supported by the fact that the affinities of orthosteric ligands for the α_{2B}-AR were unaltered upon MTα pre-incubation as already stated above (I, II). These effects seen on orthosteric ligand affinity are not in line with an allosteric model where the affinity of a ligand for the orthosteric binding site changes as a result of allosteric interaction with another ligand (65). Likewise MTα, MT3 was incapable of having effects on radioligand binding kinetics (III).

4.2.1 Live cell assays to clarify non-competitive binding (II)

As the non-competitive binding of MTα and thus the incomplete displacement of radioligands appeared not to be due to an allosteric interaction between the receptor and toxin, the partial displacement of orthosteric ligands from the α_{2B}-AR was speculated to occur as a result of fractional occupancy of receptors. This fractional occupancy might derive from receptor subspecies binding the toxin differently possibly due to receptor dimerization or incompletely processed receptors. To study this, the blocking efficiency of MTα on surface receptors was evaluated by using live cells (II). Results revealed that receptor responses were after toxin exposure much smaller than what could have been expected from the orthosteric binding sites available (Figure 17A-C). Thus, it seems that receptors not blocked by the toxin are not able to create decent responses suggesting that MTα might have effects on the efficacy of the remaining ligand-receptor complexes.

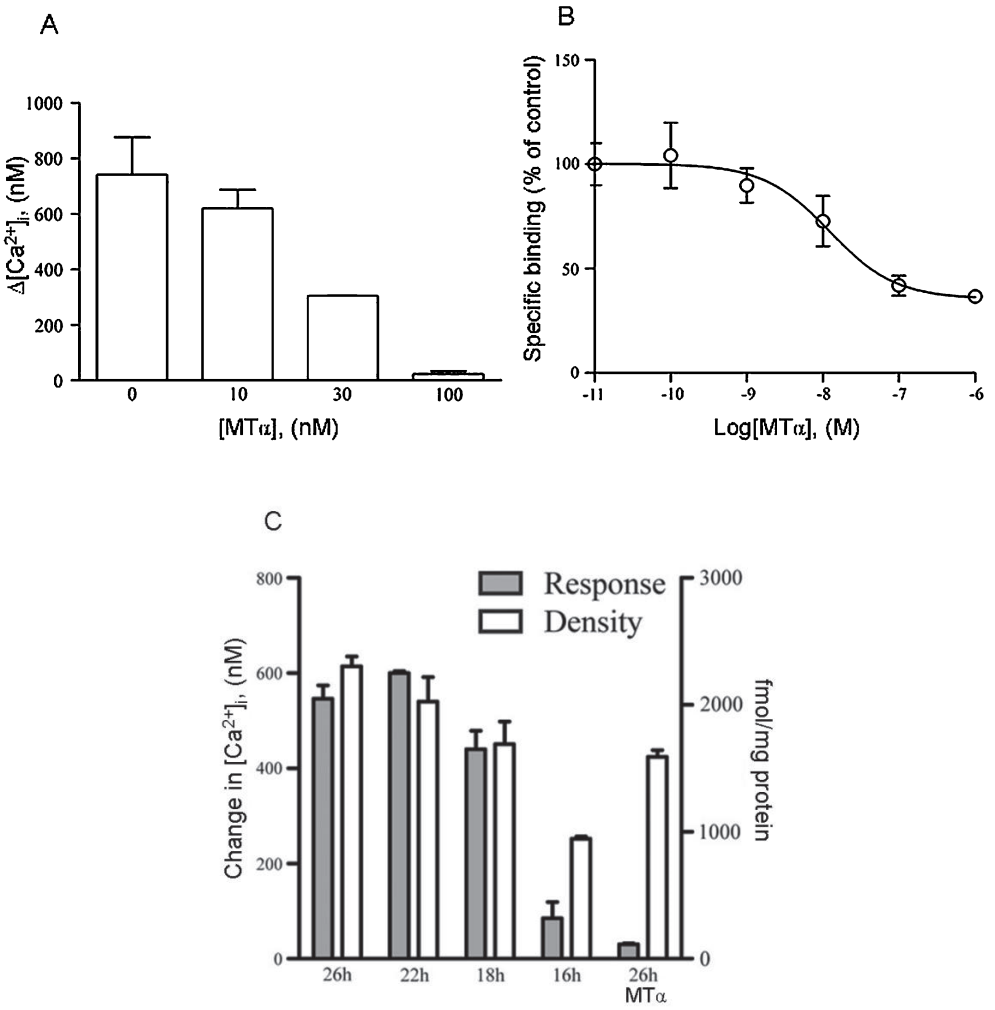

Figure 17. The blocking efficiency of MTα on surface receptors. A. Cells expressing the α_{2B}-AR were loaded with fura-2 and Ca^{2+} responses measured in the absence and presence of different concentrations of MTα. UK-14,304 was used to stimulate the receptors. B. The effect of MTα on [^{3}H]-MK-912 binding to live Sf-9 cells was measured. C. The α_{2B}-ARs were expressed for different times (16-26 h) in Sf-9 cells and the cellular responses to 10 μM UK-14,304 and surface receptor densities as detected with 3nM [^{3}H]-MK-912 were measured simultaneously. The effect of 100 nM MTα was evaluated on receptors expressed for 26 h. Data are means ± S.D. from at least one representative experiment with three triplicates. Reprinted with permission from publication II.

The binding mode of MTα to the α_{2B}-AR appears to be rather complex and it is difficult to explain with the current pharmacological concepts. However, the binding mode is similar to the effect of risperidone on serotonin 5-HT7 receptors. Risperidone binds pseudo-irreversibly only to 50 % of serotonin HT7 receptors expressed in human embryonic kidney 293 cells although all receptor activity are blocked by it (288). It was later demonstrated that the effect of risperidone on the serotonin HT7 receptors is due to homodimerization of serotonin 5-HT7

receptors (289). In this homodimeric model risperidone binds only to one protomer of the homodimeric receptor and impairs receptor function still allowing ligand binding to the other protomer. The effect of MTα on the α_{2B}-AR could be explained by this homodimeric model as illustrated in Figure 18. According to this model, MTα binds tightly to one protomer of the α_{2B}-AR homodimer changing the conformation of the receptor in such a way that coupling to a G-protein would not be possible and agonists would thus not be able to activate the receptors as seen in functional calcium mobilization assays. This conformational change could also prevent MTα from binding tightly to the other promoter allowing orthosteric ligands to compete with MTα on binding to the receptor and explaining thus the partial displacement seen in radioligand binding assays. The M_1 mAChR has been reported to form homodimers to which MT7 can bind (80, 274). This verifies that MTs have the ability of interact with dimerized receptors. The homodimeric model, however, does not exclude the possibility that MTα could also bind to monomeric form of the α_{2B}-AR. Additional studies need to be performed before the binding of MTα to the homodimeric form of α_{2B}-AR can be verified and the binding mode fully assessed.

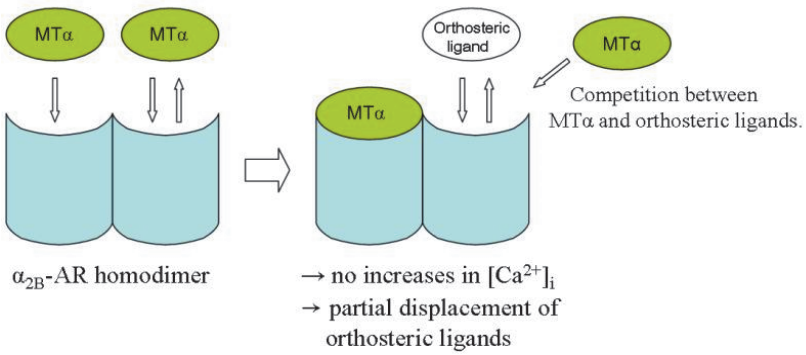

Figure 18. Schematic presentation of the homodimeric model with the effects of MTα on the α_{2B}-AR. MTα binds to one protomer of the α_{2B}-AR dimer resulting in a pseudo-irreversible complex between the toxin and receptor. This interaction precludes G-protein coupling to the α_{2B}-AR and therefore no increases in $[Ca^{2+}]_i$ can be observed. The second protomer, however, retains the ability to bind orthosteric ligands, both agonists and antagonists.

4.2.2 Complex interaction between MT3 and M_4 mAChR (III)

MT3 has been previously reported to function as a competitive antagonist of M_4 mAChR action (255, 277, 290). This assumption is based on the complete displacement of radioligands and shifts in their K_d values in the presence of MT3 (254). Yet, binding of MT3 to the M_4 mAChR seems to be a bit more complex as our studies showed that MT3 binds to the M_4 mAChR in a non-competitive manner (III). Additionally, initial shifts in the potency of MT3 were observed at low radioligand concentrations indicating thus a competitive

interaction (III). These shifts were not, however, obvious at higher radioligand concentrations, a feature which is in line with a previous report (253). The interaction of MT3 with the M_4 mAChR thus appears to be more complex than simple competition between the toxin and orthosteric ligand. As illustrated by MTα and also with MT3, it can be difficult to categorize muscarinic toxins into defined pharmacological agents. One factor contributing to this is the reversibility of interaction. Some muscarinic toxins are easily washed away from the receptor such as MT3 whereas the others such as MT7 and MTα come off very slowly which can have effects on the outcome. Another factor that should be taken into account is the existence of potential receptor reserves which can affect the inhibition profile of toxins. This was demonstrated to occur with α_{1A}-AR and MT3 (III). In cells expressing high levels of α_{1A}-AR, long pre-incubations with MT3 did not show remarkable insurmountable inhibition. On contrary to this, the agonist induced responses were strongly inhibited by MT3 with cells expressing α_{1A}-AR at low density (see publication III Figures 5A and B). This indicates that in cells expressing high levels of α_{1A}-AR, there is a receptor reserve which can elicit a full response irrespective of toxin application.

4.3 Receptor domain responsible for MTα binding (II)

The transmembrane regions of α-ARs are highly conserved. The extracellular loops on the other hand show much more divergence and are thus considered to represent potential binding sites for various ligands. For example, the amino acid differences observed in the ECL2 of α_1-ARs and α_2-ARs have been shown to have influences on their antagonist binding specificity (70, 74, 291). However, the amino acids residing outside the binding cavity can also have significant effects on the ligand binding affinities, presumably through some conformational rearrangements of the receptor (292, 293).

The primary binding sites for MT7 on the M_1 mAChR are on the ECL2 and ECL3 of the M_1 mAChR (79). The amino acids responsible for the interaction are mainly negatively charged residues in the ELC2 and 3 together with a tyrosine residue from the ECL2 (79, 270). The involvement of ECL2 in MT7 binding has been confirmed with a model of dimeric M_1 mAChR in complex with MT7. This model shows that MT7 is flanked by the ECL2 of both monomers, pointing out the crucial role of this loop for the interaction with MT7 (80). To map the major interaction site for MTα on the α_{2B}-AR, several chimeric α_2-AR constructs were designed. As α_{2A}-AR and α_{2C}-AR are not target receptors for MTα, the chimeric receptors constructs were based on these receptor sequences fused with the α_{2B}-AR. Figure 19 shows the schematic presentation of the chimeric receptor constructs. The chimeric receptor constructs were designed to reveal potential binding sites in the N-terminal part and in the ECLs 1-3.

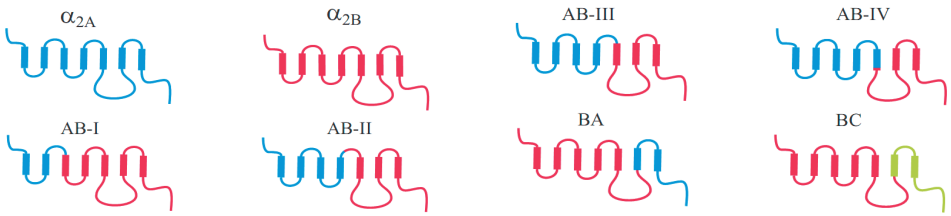

Figure 19. Schematic presentation of the chimeric receptor constructs. Blue color stands for the α_{2A}-AR, red for the α_{2B}-AR and green for the α_{2C}-AR. The figure is adapted and reprinted with permission from Figure 6 in publication II.

Chimera AB-I was used to assess the involvement of the N-terminal part and the ECL1 of the α_{2B}-AR in the binding of MTα. This chimera displayed similar affinity for MTα as the intact α_{2B}-AR (Table 7) indicating that the ELC1 and the N-terminus do not possess amino acids which take part in MTα binding. As the ECL2 has been shown to participate in ligand binding in rhodopsin-like GPCRs (16, 74-78), special emphasis was put on this loop. Three chimeras, AB-II, AB-III and AB-IV were designed so that the whole ECL2 of the α_{2B}-AR would gradually be exchanged with that of α_{2A}-AR. The sequential exchange of α_{2B}-AR ECL2 sequence with that of α_{2A}-AR gradually lowered the affinity for the toxin. The AB-II showed approximately fourfold decrease in affinity whereas a 25-fold decrease was observed with the AB-III (Table 7). The AB-IV chimera which has α_{2A}-AR sequence from the distal end of ECL2 until intracellular regions of TM5, was no longer inhibited by MTα (Table 7).

Multiple contacts between ECL2 and MTα seem to mediate the toxin-receptor interactions. The middle portion of ECL2 appears to be especially important for MTα binding as a 25-fold decrease in affinity is observed with AB-III chimera. Regarding the loss of binding affinity for MTα in AB-IV chimera, several amino acids in TM5 have been indicated to take part in ligand binding and to provide binding sites for classical ligands. Particularly important amino acids in the α_{2A}-AR are Ser200, Cys201 and Ser204 which have been linked to receptor activity and ligand binding (72, 73, 148). Of these three amino acids, Cys201 plays a significant role in the binding of catecholamine ligands and UK-14,304 (73, 294). In the human α_{2B}-AR, the amino acid corresponding to Cys201 of the human α_{2A}-AR is Ser177 at the beginning of TM5. Regarding the prominent role of Cys201 in ligand binding in the α_{2A}-AR, it might thus be that Ser177 displays a similar role in the α_{2B}-AR. It could be possible that this amino acid partly determines the subtype selectivity of MTα for the α_{2B}-AR because both α_{2A}-AR and α_{2C}-AR have cysteines in the corresponding position and are not affected by MTα.

To further investigate which amino acid in the ECL2 might contribute to loss in binding affinity for MTα, two amino acids in the intact α_{2B}-AR, Asp153 and Gln154, were mutated to alanines. Substituting Asp153 with an alanine resulted in a mutant which had slightly higher

affinity for MTα as the intact α_{2B}-AR. On the contrary to Asp153Ala mutant, the Gln154Ala mutant showed a slight decrease (2-fold) in the affinity for MTα indicating that this amino acid has a minor role in MTα binding. Considering the small effect on MTα binding affinity, it might be that Gln154 provides an additional anchoring point for MTα which is not however, significant for the toxin-receptor interactions. On the contrary to Gln154Ala mutant, the slightly higher affinity of Asp153Ala mutant for MTα is probably due to differences in the side chains of aspartic acid and alanine. Due to smaller side chain of alanine, MTα could move deeper into the binding cavity and make contacts with additional residues.

In the ECL3 of M_1 mAChR, there is one amino acid, Glu397, which has been speculated to take part in MT7 binding (79). It could be that this negatively charged amino acid interacts with positively charged amino acids present in MT7 sequence. For this reason it was examined if there were sites in the ECL3 of α_{2B}-AR that would participate in MTα binding. For this purpose BA and BC chimeras were created. In these chimeras the ECL3 of α_{2B}-AR was exchanged with either of α_{2A}-AR or α_{2C}-AR sequence. Both BA and BC chimeras had similar affinities for MTα as the intact α_{2B}-AR indicating that ECL3 does not have amino acids participating in MTα binding (Table 7).

Table 7. Inhibitory potencies of MTα on the receptor constructs.

Receptor construct	pIC_{50} for MTα	IC_{50}, mod/IC_{50}, intact*
α_{2B}	8.64 ± 0.14	
AB-I	8.63 ± 0.07	1
AB-II	7.99 ± 0.08	4.5
AB-III	7.25 ± 0.16	24.5
AB-IV	-	-
BA	8.51 ± 0.08	1.3
BC	8.46 ± 0.15	1.5
α_{2B}-Asp153Ala	8.89 ± 0.05	0.6
α_{2B}-Gln154Ala	8.32 ± 0.15	2.1

Values are means ± S.E.M. (n = 3-4). *The IC_{50}, mod/IC_{50}, intact-values were obtained by dividing the IC_{50} values of the modified receptors with that of the intact (unmodified) α_{2B}-AR. By this way the IC_{50}, mod/IC_{50}, intact-value for the intact α_{2B}-AR is 1.

Currently it not known how deep into the binding cavity muscarinic toxins can penetrate. Although muscarinic toxins are rather large molecules (~ 8 kD), in theory it is possible that one of their three fingers reaches down into the binding cavity as suggested by Segalas and co-workers (271). Yet, peptides tend to bind to the extracellular sequences and N-terminus of GPCRs although the size of the ligand does not necessarily always indicate the actual binding site (68). A model of dimeric M_1 mAChR in complex with MT7 demonstrated that MT7 is flanked by ECL2 of both protomers of dimerized receptor suggesting that MT7 does not penetrate into the ligand binding cavity (80, 274). This is in line with the observation

regarding the binding site for large peptide ligands. The different loops of MT7 make contacts with different protomers of the dimerized M_1 mAChR; MT7 loops II and III interact with protomer A and MT7 loop I with protomer B (80). Like MT7, MTα might also preferably interact with a dimerized α_{2B}-AR through the ECL2. In such a scenario, MTα binding to one α_{2B}-AR protomer could constrain the ECL2 into an unfavorable conformation resulting eventually in a situation where dissociation of MTα from the receptor becomes extremely slow (pseudo-irreversible). This conformational change could also prevent orthosteric ligand binding to this protomer while still allowing both toxin and orthosteric ligand binding to the other protomer.

4.4 Technical applications (IV)

Peptide toxins used in research do not normally exist at the plasma membrane. However, several venom toxins have been successfully linked to the plasma membrane via a GPI-tail. Anchoring toxins to the plasma membrane puts them in close vicinity of their target receptors or ion-channels and allows cell-specific silencing of cellular responses without interfering with the signaling of the neighboring cells (295, 296). This strategy has also been applied for some peptide ligands to induce constitutive activation of GPCRs (297, 299) indicating that this approach can be successfully adapted also to other bioactive peptides. All the toxins and peptides anchored to the plasma membrane so far have retained their activity and specificity for their target receptors and ion channels.

The three-finger receptor toxins are evolutionary related to the Ly-6 antigens of the immune system (220, 221). Some members of the Ly-6 antigen family, such as the endogenous nAChR modulator lynx1, are attached to the plasma membrane via a GPI-tail (222). In fact, lynx1 was the first membrane-bound peptide found to affect the function of neuronal nAChRs (222). The consequence of such an interaction is that the nAChRs bound with lynx1 are less sensitive to their agonists and desensitize more rapidly (223). The applicability of GPI-anchoring to MTs was evaluated in the fourth publication (IV). Addition of a GPI-tail to MTs should direct the toxins to the secretory pathway of the cell and eventually target them to the plasma membrane. For the study three GPI-anchored toxins were created; MT7-GPI, T3L-GPI which differs from MT3 sequence by two amino acids and Tα/1L-GPI which differs from MTα and MT1 sequences only in few positions (see publication IV Figure 1).

All the GPI-anchored toxins created were able to block agonist induced receptor responses when expressed in Sf9 cells together with their target receptors (Figure 20A, B and C). MT7-GPI did not interfere with signaling resulting from carbachol binding to M_3 mAChRs (Figure 20D) but it was able to almost fully inhibit the specific [^{3}H]-NMS binding to M_1 mAChRs while no effect on the M_4 mAChRs was observed (see publication IV Figure 4). This indicates

that MT7-GPI has the same receptor specificity as soluble MT7. Likewise MT7-GPI, the T3L-GPI behaved as soluble MT3 (see publication IV Figure 4). The Tα/1L-GPI blocked α_{2B}-AR induced responses as soluble MTα but showed reduced binding affinity for the M_1 and M_4 mAChRs which is different from the moderate binding affinity of MT1 (pIC_{50} = 6.5-7) for these two mAChR subtypes (258, 261). Compared to MTα sequence, Tα/1L-GPI-construct has a proline in position 33 like MT1. As previously discussed in section 4.2, Pro33 might have a role in mAChR binding in MT1 as MTα does not show binding to these receptors (I).

Based on the results it can be concluded that GPI-anchor can be added to muscarinic toxins without interfering with toxin binding to the receptors. Membrane anchoring did not interfere with receptor trafficking to the plasma membrane as receptors were found to be localized at the membrane irrespective of simultaneous toxin expression (Figure 21). This was shown with the M_1 mAChR tagged with enhanced Green Fluorescent Protein (eGFP) and co-expressed with either soluble MT7 or MT7-GPI (Figure 21).

Anchoring peptides and toxins to the plasma membrane is an innovative technique for modulating the biological function of receptors and ion channels both *in vitro* and *in vivo*. It helps to increase the cellular specificity of toxins by restricting their actions only to certain cells. Membrane anchored MTs can be applied to basic research to study physiological roles of ARs and mAChRs more closely in various tissues, especially in the CNS, organs and even in individual cells depending on the selectivity of MTs. Additionally membrane anchored MTs could also be used to block receptor activity in disease conditions such as in primary hypertension resulting from prolonged activation of α-ARs (158). An example of such an activity block is presented by α-BTx and nAChRs. The ion currents via nAChRs in muscle cells were efficiently blocked *in vivo* by membrane anchored α-BTx using a transgenic approach on zebrafish (295). Muscle cells not expressing membrane anchored toxins remained normal (295). Membrane anchored approach offers also a possibility to use MTs in the design of new therapeutics for ARs and mAChRs as soluble toxins have some major limitations such as scarcity in venoms and lack of cell-selectivity. Overall, anchoring MTs to the plasma membrane is relatively easy to put into practice but it offers major potential for both the basic research and pharmaceutical industry.

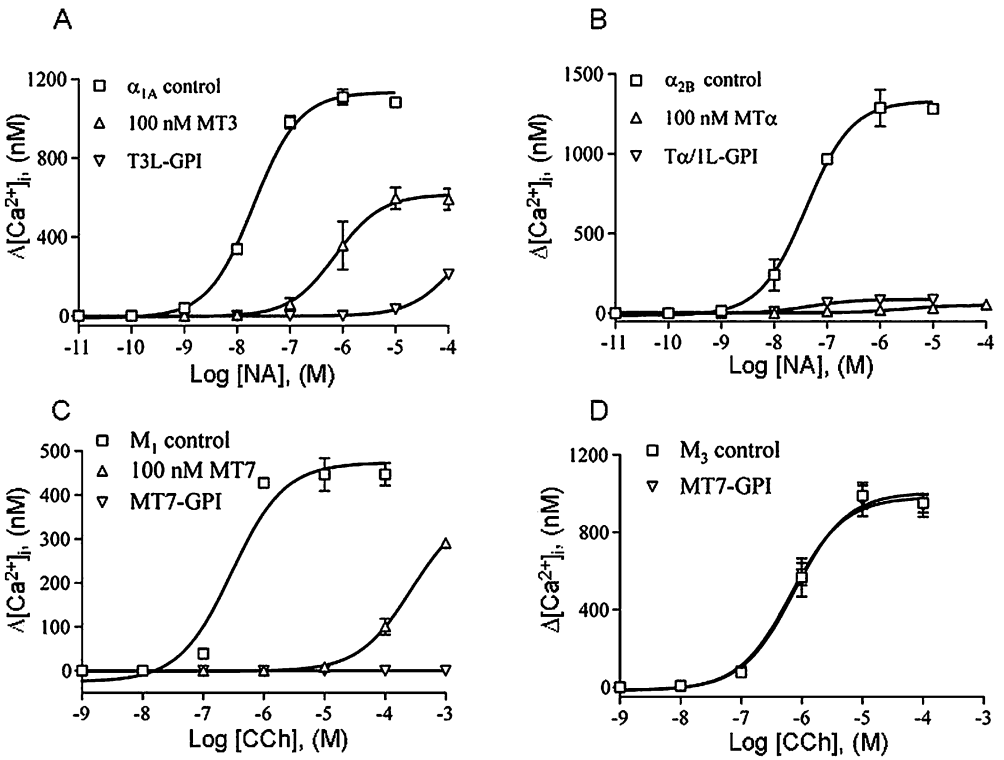

Figure 20. Effect of GPI-anchored muscarinic toxins on the mAChRs and α-ARs. Cells expressing receptors alone or co-expressing receptors and GPI-anchored toxins were assayed for noradrenaline (NA) (A and B) or carbachol (CCh) (C and D) induced increases in $[Ca^{2+}]_i$. Control cells were also tested with soluble MTs for comparison. Data points are means ± SD from three experiments. The figure is adapted and reprinted with permission from figures 2 and 3 from publication IV.

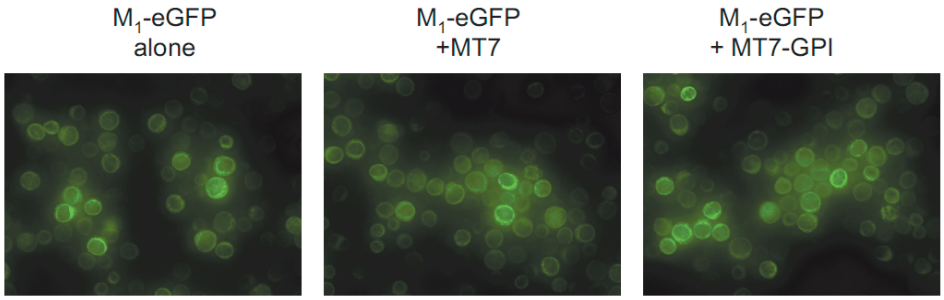

Figure 21. Fluorescence images of eGFP-tagged M_1 mAChRs. Sf9 cells were infected with M_1-eGFP virus alone or together with MT7 (secreted, soluble) or MT7-GPI for 27 h.. Fluorescence microscopy was performed using a Carl Zeiss Axiovert 200M inverted microscope (Carl Zeiss Inc., Jena, Germany) with a 40×/0.6 objective. The figure is reprinted with permission from publication IV (supplementary Figure S1).

5 CONLUSIONS

Subtype selective ligands are important both for the basic biological research and for the pharmaceutical industry. For the ARs most of the ligands in current use are rather unselective which puts limits on their use. In this thesis we examined the ability of muscarinic toxins to function as new receptor ligands for the ARs. Muscarinic toxins appeared to be multifunctional three-finger toxins targeting both mAChR and α-AR subtypes. Additionally these toxins could be anchored to the plasma membrane to obtain cell-specific inhibition of receptor responses.

Among the toxins studied MT7 and MTα were shown to be highly selective for their target receptors, M_1 mAChRs and α_{2B}-ARs, respectively, whereas MT1 and MT3 had a broad range of target receptors. Regarding MTα, this is the first peptide ligand known to act on the α_{2B}-ARs. The interaction between the α_{2B}-AR and MTα is rather complex but it shows clear signs of non-competitiveness. Considering MTα binding and its inhibitory effects on receptor function, it is likely that MTα binds to a dimerized form of the α_{2B}-AR through the second extracellular loop. However, more studies need to be performed to clarify this.

The ability of MTs to interact with both ARs and mAChRs opens up new possibilities for the pharmaceutical industry but also for the basic biological research. These toxins can be used as pharmacological tools to study AR physiology more closely in the organs they affect. They also seem to be useful as three-dimensional templates to develop molecular mimics which can later be used to treat several diseases such as cardiovascular diseases and benign prostatic hyperplasia. Additionally, these toxins, especially MT3, could be used to develop more subtype selective ligands since α-ARs and mAChRs are still lacking them. The ability to anchor these toxins to the plasma membrane with a GPI-tail is also relevant. This approach offers several possibilities; it can be used for example to examine the function of ARs and mAChRs *in vivo* in model organisms and to cell specifically inhibit receptor functions in specific cells.

In conclusion, this thesis provides new tools to tackle the problem associated with the lack of subtype selective ligands for the adrenoceptors and mAChRs. It also leads to better understanding of toxin action on ARs and mAChRs.

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