

**Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 183.**

**Muscarinic Toxins from
Dendroaspis (Mamba) Venom**

*Peptides Selective for Subtypes of
Muscarinic Acetylcholine Receptors*

BY

MIKAEL JOLKKONEN

**ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 1996**

Doctoral dissertation for the degree of Doctor of Philosophy in Biochemistry presented at Uppsala University, 1996

Abstract

Jolkkonen, M., 1996. Muscarinic Toxins from *Dendroaspis* (Mamba) Venom: Peptides Selective for Subtypes of Muscarinic Acetylcholine Receptors. Acta Univ. Ups., *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 183. 52 pp. Uppsala. ISBN 91-554-3675-7

Snake venoms from various mamba species contain peptides which bind to the muscarinic but not to the nicotinic acetylcholine receptors. Peptides with this function have not been isolated from any other organism. These peptides were called muscarinic toxins, although the toxicity of the purified peptides is very limited.

A number of muscarinic toxins were isolated from venom of the Eastern green mamba *Dendroaspis angusticeps* and the black mamba *D. polylepis*. The sequences of seven muscarinic toxins were determined. They contain 65 or 66 amino acids and four disulfides, and are homologous to other snake toxins such as fasciculins, α -neurotoxins, κ -neurotoxins and cytotoxins.

Studies of the binding the muscarinic toxins to cloned human receptors of subtype m1 through m5, expressed in Chinese hamster ovary (CHO) cells, revealed that several of the toxins have a high degree of subtype selectivity, up to 10 000-fold for a single subtype over others. Attempts to synthesise subtype-selective ligands have consistently met with very limited success, and the superior selectivity of the muscarinic toxins renders them valuable tools in receptor studies. This was exemplified by the use of radioiodinated muscarinic toxin 1 (MT1) to autoradiographically investigate the distribution of m1-receptors in brain. The method gave a narrower distribution of binding than the less selective, commonly used ligand pirenzepine.

Sequence comparison of muscarinic toxins with different selectivity allowed the identification of one region, residues 31–33, crucial for the selective binding to receptor.

The affinities of the toxins varies from 200 pM for the preferred subtype to several μ M for the less favoured subtypes. Different toxins not only have very different affinities and selectivities, but also different binding rates. Studies of the binding kinetics revealed that the binding includes a fast and readily reversible association step followed by the slow formation of a more stable toxin-receptor complex. In contrast to well-documented results on the related m1-toxin from *D. angusticeps*, no allosteric effects on antagonist binding could be observed with *D. angusticeps* toxin MT3.

Preliminary investigations indicate that the toxins have agonistic effects. This is supported by results of other workers in the field, again with the exception of m1-toxin. Thus there seems to exist qualitative as well as quantitative differences between individual muscarinic toxins.

The structure-function relationship of the toxins is discussed, and it is concluded that the toxins are likely to find use as biochemical probes, and hopefully also in studies of receptor function and in ligand design.

Key words: Snake venom, snake toxin, sequence determination, muscarinic acetylcholine receptor, CHO cells, ligand binding, binding kinetics, autoradiography.

Mikael Jolkkonen, Department of Biochemistry, Biomedical Centre, Box 576, S-751 23 Uppsala, Sweden.

© Mikael Jolkkonen 1996

ISSN 1104-232X
ISBN 91-554-3675-7

Printed in Sweden by Uppsala University, Reprocentralen, HSC, Uppsala, 1996.

This thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. Karlsson, E., Risinger, C., Jolkkonen, M., Wernstedt, C. and Adem, A. (1991)
Amino acid sequence of a snake toxin that binds to the muscarinic acetylcholine receptor.
Toxicon **29**, 521-515.
- II. Jolkkonen, M., Adem, A., Hellman, U., Wernstedt, C. and Karlsson, E. (1995)
A snake toxin against muscarinic acetylcholine receptors: amino acid sequence, subtype specificity and effect on guinea-pig ileum.
Toxicon **33**, 399-410.
- III. Karlsson, E., Jolkkonen, M., Satapyan, N., Adem, A., Kumlin, E., Hellman, U. and Wernstedt, C. (1994)
Protein toxins that bind to muscarinic acetylcholine receptors.
Ann. N.Y. Acad. Sci. **710**, 153-161.
- IV. Vandermeers, A., Vandermeers-Piret, M-C., Rathé, J., Waelbroeck, M., Jolkkonen, M., Oras, A. and Karlsson, E. (1995)
Purification and sequence determination of a new muscarinic toxin (MT4) from the venom of the green mamba (*Dendroaspis angusticeps*).
Toxicon **33**, 1171-1179.
- V. Jolkkonen, M., van Giersbergen, P.L.M., Hellman, U., Wernstedt, C. and Karlsson, E. (1994)
A toxin from the green mamba *Dendroaspis angusticeps*: amino acid sequence and selectivity for muscarinic m4 receptors.
FEBS Letters **352**, 91-94.
- VI. Jolkkonen, M., van Giersbergen, P.L.M., Hellman, U., Wernstedt, C., Oras, A., Satyapan, N., Adem, A. and Karlsson, E. (1994)
Muscarinic toxins from the black mamba *Dendroaspis polylepis*.
Eur. J. Biochem, in press.
- VII. Toomela, T., Jolkkonen, M., Rinken, A. And Karlsson, E. (1994)
Two-step binding of green mamba toxin to muscarinic acetylcholine receptor.
FEBS Lett. **352**, 95-97.
- VIII. Jolkkonen, M., Järv, J., Toomela, T. and Karlsson, E. (1995)
Isomerisation of complex between black mamba toxin MT α and muscarinic receptor.
Manuscript.
- IX. Adem, A., Jolkkonen, M., Bogdanovic, N., Islam, A. and Karlsson, E. (1995)
Localization of M₁ muscarinic receptors in rat brain using selective muscarinic toxin-1 (MT-1).
Manuscript.

ABBREVIATIONS.....	6
INTRODUCTION.....	7
BACKGROUND TO THE PRESENT WORK.....	7
An outline of the cholinergic system.....	7
Excitable membranes and the action potential.....	8
Acetylcholine receptors: their function and distribution.....	9
Nicotinic acetylcholine receptors	9
Muscarinic acetylcholine receptors	10
Ligands to muscarinic acetylcholine receptors.....	13
Snakes and their venoms.....	14
The mambas.....	14
Composition of snake venoms.....	15
SCOPE OF THE PRESENT WORK	19
INTRODUCTION TO THE METHODS USED.....	20
Isolation of muscarinic toxins: Separation strategy	20
Binding studies.....	21
Basic concepts in ligand binding	21
Measuring the binding of toxins.....	22
Sources of muscarinic receptors.....	24
DISCUSSION OF THE INDIVIDUAL PAPERS.....	26
Characterisation of the first muscarinic toxins: Papers I, II and III	26
The first isotoxin: Paper IV.....	30
Binding studies using cloned receptors : Papers V and VI.....	30
The kinetics of toxin binding: Papers VII and VIII.	35
Autoradiographic mapping of receptors with radiolabelled toxin MT1: Paper IX.....	38
CONCLUSIONS.....	40
ACKNOWLEDGEMENTS	42
ERRATA.....	44
APPENDIX	45
REFERENCES	46

ABBREVIATIONS

A ^M	molar absorption
AMP	adenosine monophosphate
CHO	Chinese hamster ovary
<i>e. g.</i>	<i>exempli gratia</i> = for example
GABA	4-aminobutanoic acid (earlier γ -aminobutyric acid)
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
<i>i. e.</i>	<i>id est</i> = that is to say
IC ₅₀	concentration giving 50% inhibition
K _d	dissociation constant
K _i	inhibition constant
LD ₅₀	dose lethal for 50% of a population
Mr	relative mass
MT	muscarinic toxin
NMS	N-methylscopolamine
pK _i	negative logarithm of the dissociation constant K _i
QNB	quinuclidinyl benzilate

Abbreviations used for amino acids:

Amino acid	Three-letter code	One-letter code
Aspartic acid	Asp	D
Asparagine	Asn	N
Aspartic acid or asparagine	Asx	B
Threonine	Thr	T
Serine	Ser	S
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glutamic acid or glutamine	Glx	Z
Proline	Pro	P
Glycine	Gly	G
Alanine	Ala	A
Cysteine	Cys	C
Valine	Val	V
Methionine	Met	M
Isoleucine	Ile	I
Leucine	Leu	L
Tyrosine	Tyr	Y
Phenylalanine	Phe	F
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Tryptophan	Trp	W

INTRODUCTION

This thesis describes the isolation and characterisation of a new group of snake toxins, which bind to the muscarinic acetylcholine receptors. These so called muscarinic toxins are found exclusively in the venoms of snakes of the mamba family. Mamba venoms contain a large number of different neurotoxic components, some of which are closely related to toxins from other snake venoms. In order to address the subject of muscarinic toxins in its proper context, a brief review of snake toxins in general and the mechanisms of neurotoxicity is presented.

In many textbooks of general biochemistry, the term "acetylcholine receptor" is used more or less synonymously with "nicotinic acetylcholine receptor", while the other main class, muscarinic acetylcholine receptors, is only briefly mentioned. For this reason, a short presentation of both types of receptors is included in this thesis, with an emphasis on the various subtypes of muscarinic acetylcholine receptors and the more common ligands used in their study. Some methods which find application mainly in the study of ligand-receptor interactions and may be less familiar to workers in other areas of biochemistry are briefly commented upon.

BACKGROUND TO THE PRESENT WORK

An outline of the cholinergic system.

Nerve signals have to be transmitted over macroscopic distances with speed and precision. Many of the cells involved, like neurons and muscle cells, have exceptionally elongated structures (axons and myofibers) which enhances the performance. Still, a signal propagated only by longitudinal diffusion through these cells would take very long time to reach from one end of the axon to the other, and more rapid mechanisms are needed. The signals also need to be rapidly and accurately transmitted from one cell to another, and the system must allow fast relaxation and repeated signalling. Consequently, several highly specialised mechanisms have evolved to enable efficient signal transmission.

For the present purpose, it is sufficient if we limit the discussion to the cholinergic system. In this system, the signals are transmitted by an electrophysical chain reaction within the cells, and by diffusion of the signal substance acetylcholine over the short distances between cells. This was once assumed to be the most important signal system centrally as well as peripherally. It is now believed that the fast synaptic transmission in brain is mediated by other signal substances, such as glutamate. These other signalling systems, although closely interconnected with the cholinergic system, will not be discussed at any greater length in this treatise.

Excitable membranes and the action potential

The nerve signals are of an electrical as well as chemical nature, and the so called excitable membranes of nerve and muscle cells play a crucial role in the propagation of these signals. These membranes are nearly impervious to most ions and molecules, which can cross the membrane only by way of certain channels and pores, sometimes by diffusion (along the concentration gradient, often called "downhill"), in other cases by active transport (against the concentration gradient, or "uphill"). The concentration gradient is maintained by "uphill" transport, which requires energy and results in an accumulation or depletion of a chemical species inside the cell relative to the outside. An unequal distribution of an- and cations is an uneven distribution of electric charge, in other words, such a chemical gradient, or potential, is accompanied by an electric potential across the membrane. In the resting, or unstimulated, axon the electric potential relative to the outside is -60 mV in the postsynaptic membrane and about -75 mV at the motor endplate.

A nerve signal is triggered when the membrane becomes depolarised, *i. e.*, the membrane potential rises above the threshold value of about -40 mV. This leads to the opening of the voltage-regulated sodium-specific channels. These channels are also called voltage-gated or voltage-dependent sodium channels, and assume a "closed" or an "open" conformation depending on the membrane potential. The positively charged sodium ions then flow into the cell until the potential has increased to about +30 mV relative to the outside, at which time the driving concentration gradient is cancelled out by the opposing voltage gradient. The swift depolarisation leads to the opening of neighbouring sodium channels, and the signal is thus propagated along the axon. Meanwhile, the sodium channels spontaneously close and potassium channels open. Potassium ions flow out from the cell until the voltage gradient halts the process at a potential of about -75 mV. With the closing of the potassium channels, the potential quickly stabilises at the resting potential of -60 mV. The whole cycle takes only a few milliseconds. This self-propagating wave of depolarisation is called the action potential. Due to the spontaneous closing of the sodium channels, it is unidirectional; the action potential does not reverse its direction of travel but terminates at the end of the axon, where it causes voltage-regulated calcium channels to open. Calcium ions then enter the cytosol and initiate a process by which vesicles filled with the signal substance acetylcholine fuse with the cell membrane, releasing their content to the outside. The released acetylcholine is rapidly hydrolysed to acetate and choline by the enzyme acetylcholinesterase, and the choline is mostly reabsorbed across the pre-synaptic membrane and used for synthesis of new acetylcholine.

Some of the released acetylcholine will have time to diffuse the short distance to the "target" cell. If that is another nerve cell, the structure where the cells meet is called a synapse. If it is a muscle cell, it is called the neuromuscular junction. The binding of acetylcholine to certain receptors in the target cell (at least if it is a muscle cell; see the following section for a discussion of neuronal nicotinic receptors) then allows sodium to flow across the membrane, which initiates a new action potential in the post-synaptic cell. These receptors are described as

post-junctional or post-synaptic, due to their location, and are of the type called nicotinic acetylcholine receptors. Their structure is discussed more in detail in the following section.

The ion channels, receptors and enzyme that have been briefly mentioned here exist in several forms, with different distributions, ways of regulation, and functions, and it should be kept in mind that the purpose of this simplified account is only to provide some background necessary for the discussion of neurotoxins.

Acetylcholine receptors: their function and distribution.

The classification of acetylcholine receptors is a good example of how natural toxins can be used to identify and distinguish between receptors. Acetylcholine receptors were found to be of two types: the nicotinic type, which is activated by nicotine (from the tobacco plant *Nicotiana tabacum*) and blocked by tubocurarine (from the South American vine *Chondodendron tomentosum*), and the muscarinic type, which is activated by muscarine (from the fly agaric *Amanita muscaria*) and blocked by atropine (from the deadly nightshade *Atropa belladonna*) (Dale, 1914). Nicotinic receptors are found at high concentration in the motor endplates (the postjunctional membrane of the neuromuscular junction) of vertebrate skeletal muscle, and to a smaller extent in the prejunctional membrane, the autonomic ganglia, and in the central nervous system of both vertebrates and invertebrates.

Nicotinic acetylcholine receptors

The nicotinic acetylcholine receptors consist of five homologous subunits, $\alpha_2\beta\gamma\delta$, where the α subunits have one binding site each for acetylcholine. The subunits are transmembrane proteins and together form a pentagonal pore. In the postsynaptic membrane the nicotinic receptors are very closely packed together, and form dimers with the pentameric receptors bonded together in pairs by disulphide bonds between the δ subunits (McCrea *et al.*, 1987; DiPaola *et al.*, 1989). It is unclear whether the receptors form dimers also when less densely packed.

On the binding of acetylcholine to both α subunits, the pore opens and becomes permeable to cations. The nicotinic acetylcholine receptor thus functions as an acetylcholine-regulated (or ligand-gated) channel. In contrast to the voltage-regulated sodium channels, it readily allows both sodium and potassium ions to pass (Mullins, 1975), but because of the electrochemical gradient, the main effect of the opening of the channel is an influx of sodium ions, leading to depolarisation of the membrane and initiation of an action potential.

The nicotinic acetylcholine receptors in muscle are distinct from their neuronal counterparts, which in their turn are divided into central and ganglionic and fall into a number of subclasses. The above mentioned subunits (α to δ) exist in a number of variants; for instance, at least nine variants of neuronal α subunits are known, and are divided into two classes based on their structural similarity to α subunits in muscle. The non-alpha subunits of neuronal nicotinic receptors do not form distinct classes of β , γ , and δ subunits but are more or

less interchangeable with each other (Sargent, 1993), and are commonly all called β and given an index to differentiate between them. The complexity of neuronal nicotinic receptors is illustrated by the fact that basic textbooks remain remarkably silent on the subject, concentrating on the much better known muscular nicotinic receptors. A full account of the classification of nicotinic receptors, if such a thing was possible, would still not be within the scope of this thesis, and it may suffice to say that the research in this field is likely to continue for many years more before the structure and function of all nicotinic receptors is understood - assuming that they all have a function, which has been questioned (Sivilotti and Colquhoun, 1995). Recently, it has been found that mice lacking functional central nicotinic receptors appear to be perfectly healthy and differ from the wild-type only in that they perform better than their wild-type relatives in a learning task, and that their performance is insensitive to nicotine (Picciotto *et al.*, 1995), and it has been suggested that central nicotinic receptors mainly or exclusively regulate the excitability of neurons rather than mediate the transmission of the nerve signals (McGehee *et al.*, 1995; see also the following section).

Muscarinic acetylcholine receptors

The muscarinic acetylcholine receptors, structurally unrelated to the nicotinic type, are simple in comparison. They are monomeric proteins with seven hydrophobic, membrane-spanning helices, and share a common evolutionary origin with α and β - adrenergic receptors, substance K receptors, cannabinoid receptors, rhodopsin, opsin and a number of serotonin receptors. This superfamily of membrane proteins is often referred to as seven-helix receptors.

The muscarinic receptors have a binding site for acetylcholine accessible from the extracellular side and a binding site for a guanine nucleotide-binding regulatory protein (G protein) on the intracellular side. G proteins are associated with many types of receptors and regulate a large number of channels and intracellular processes. A large number of G proteins of different composition and function are known (Birnbaumer *et al.*, 1987). They all consist of three subunits called α , β and γ . Binding of acetylcholine to the muscarinic receptor probably induces a conformational change which is propagated through the membrane and allows the binding of a G-protein to an intracellular region of the receptor. The G-protein thereupon becomes activated and the α subunit dissociates from the $\beta\gamma$ dimer. The regulation is then effected either directly by the binding of an activated subunit to the target protein or indirectly by the mediation of a second-messenger system, typically involving one or several enzymatic reactions.

Since the pathway consists of several steps, which are limited by diffusion speed, binding and dissociation rates, enzyme kinetics *et cetera*, the effects of agonist binding to muscarinic receptors are slow as compared to the nicotinic receptors.

The amino acid sequences of five different muscarinic acetylcholine receptors, called m1, m2... m5, have been determined. An alternative, pharmacological classification of acetylcholine receptors into groups called M1, M2 (earlier called M2 cardiac type) and M3

(earlier called M2 glandular type) is based on the affinities of selective ligands such as pirenzepine to the receptors. The correlation between these nomenclatures is not quite straightforward, and has at times been downright confusing (see Table 1). Following the latest standard of pharmacological classification, M1 probably corresponds to m1, M2 to m2 and M3 to m3, while the m4 and m5 receptors usually are not assigned to any of the classical pharmacological groups. Genes encoding for at least four additional muscarinic receptor-like proteins have been detected by genomic blot hybridisation (Bonner, 1989), but the corresponding proteins have not yet been detected.

Table 1. Nomenclature of muscarinic acetylcholine receptors. This nomenclature was recommended by the 4th Symposium of Subtypes of Muscarinic Receptors and the British Pharmacological Society Nomenclature Subcommittee, 1989.

Pharmacological characterisation:

Recommended nomenclature	M ₁	M ₂	M ₃	–	–
Previously used names	M _{1α} A	M _{2α} cardiac M2 C	M ₂ M _{2β} glandular M2 B		
Molecular characterisation:					
Sequences	m1	m2	m3	m4	m5
Previously used names	mAChRI M1	mAChRII M2	mAChRIII M4	mAChRIV M3	
Number of amino acids	460	466	589/590	478/479	531/532

The five subtypes are expressed to different extent in different tissues. The expression of subtype m5 is extremely limited, and the function of this subtype is poorly understood. The subtypes are associated with different G proteins and second messenger systems. Muscarinic receptors of subtypes m1, m3 and m5 are associated mainly with G-proteins of type Gq/G11 (which stimulate the hydrolysis of phosphoinositol by phospholipase C, resulting in increased concentrations of the second messengers inositol triphosphate and diacylglycerol), while m2 and m4 are associated mainly with the Gi/Go type (which inhibit adenylate cyclase, resulting in decreased concentrations of the second messenger cyclic AMP). Furthermore, the same second messenger can have different effects in different tissues. Agonist binding to different classes of muscarinic acetylcholine receptors, or to the same receptors in different tissues, may therefore be expected to have different effects.

For instance, the local effect of acetylcholine binding to muscarinic receptors in the heart

(the pacemaker cells in the sinoatrial node) is a decrease in heart rate, but the central effect (in the rostral ventrolateral medulla) is an increase in heart rate (Giuliano *et al.*, 1989). In most cases activation of muscarinic receptors amplify the action potential by closure of M-type potassium channels, but in the sinoatrial node the effect is an opening of potassium channels, with inhibitory results (Sakmann *et al.*, 1983). Muscarinic receptors can mediate both contraction and relaxation of blood vessels. Other effects of muscarinic agonists are stimulated secretion from glands (*e. g.*, increased sweating and salivation, tear flow and secretion from the pancreas), and contraction of smooth muscle in the intestine, airways, urinary bladder and uterus (for review, see Caulfield, 1993). Stimulation of central muscarinic receptors (in the rostral ventrolateral medulla) also increase respiration and blood pressure (Dev and Loeschcke, 1979; Nattie *et al.*, 1989).

Muscarinic receptors also influence the cognitive processes. Poisoning with muscarinic antagonists produces symptoms such as restlessness, mania, hallucinations, confusion and delusions (Weiner, 1980; Shulgin, 1982). The effects of muscarinic agonists, especially muscarine, have been reported to be similar (Sollmann, 1957). Although poisoning with muscarinic agonists may cause confusion, their hallucinogenic effects have probably been exaggerated in the past, most likely due to the misconception that the psychological effect of the fly agaric *Amanita muscaria* is attributable to its muscarine content. While the muscarine content of the fly agaric is rather moderate, it contains several psychoactive alkaloids such as muscimol and ibotenic acid which probably answer for most if not all of its hallucinogenic effects (Taylor, 1980; Shulgin, 1982).

The psychological effects of muscarinic drugs have long been exploited for religious or recreational purposes (Shulgin, 1982; Metzner, 1970), and later in forensic medicine as a putative "truth serum" (see under "deception test" in the 1947 edition of Encyclopædia Britannica) and by the military for creating "incapacitating" chemical warfare agents (Shulgin, 1982; Meselson and Robinson, 1980). The recreational use probably requires some deeper interest in psychedelics, since there seems to be a general consensus that the effects are not purely pleasurable. It should also be noted that the legal value of the "truth serums" of different compositions is strongly disputed, since the subjects occasionally produce free fabrications. Furthermore, the US production of the muscarinic warfare agent BZ (identical to quinuclidinyl benzilate, QNB), which was intended to be a "humane weapon" that confused and thereby incapacitated the enemy without killing him, has been discontinued because it could induce a state of temporary insanity in the subjects, making their actions unpredictable and potentially more dangerous (Meselson and Robinson, 1980).

The memory is particularly sensitive to muscarinic drugs. Scopolamine poisoning induces a state resembling dementia, and has been used as a model for Alzheimer's disease. Injection of a small quantity of muscarinic agonist into the brain of mice improves their performance in learning tasks, while the antagonist scopolamine impairs it (Izquierdo, 1989).

There is much evidence for the existence of pre-synaptic autoreceptors of both the

muscarinic and nicotinic kind (for review, see Bowman, 1990). Binding of an agonist to the muscarinic autoreceptors increases the release of acetylcholine, but at higher agonist concentrations, the effect is the opposite. This may have the effect of amplifying the initial burst of transmitter, and shortly thereafter helping to terminate the impulse. Some studies suggest that the facilitatory effect is mediated by the M1 and the inhibitory by the M2 and M3 class of receptors (Goyal, 1989).

Also the release of signal substances other than acetylcholine is modulated by muscarinic receptors. Among these are dopamine, glutamate and GABA. It has been proposed that the release of dopamine from substantia nigra is regulated by the m5 subtype, whose function has been disputed (for review, see Caulfield, 1993).

Recent studies suggest that the function of the nicotinic receptors in the central nervous system is mainly or exclusively such regulation of the release of various signal substances and fine-tuning of the excitability of neurons, rather than actual mediation of nerve signals over the synapse (McGehee *et al.*, 1995).

Ligands to muscarinic acetylcholine receptors

Ligand to receptors are usually divided into agonists, which initiate a response upon binding, and antagonists, which block the receptor and make it insensitive to agonists. There also exist mixed types of ligands, having both agonistic or antagonistic effects, of which one may be predominating at low and the other at high concentrations. So called partial agonists are characterised by not producing "full" effect, no matter what concentrations are being used. They do, however, not necessarily have to bind to both agonistic and antagonistic sites. A partial agonist may for instance bind to and open an ion channel, but allow it to close spontaneously, while a "full" agonist blocks the channel in the open position. In the case of muscarinic receptors, an agonist can be defined as a molecule that mimics the effect of acetylcholine, and an antagonist as one that decreases the effect of added acetylcholine.

Many of the earliest known ligands for muscarinic acetylcholine receptors were of a natural origin, typically toxins used by plants as defence against insects and herbivores. The *Solanaceae* family, which includes plants such as deadly nightshade, Jimson weed (thorn apple), tobacco and potato, is particularly rich in muscarinic toxins, such as the closely related antagonists atropine, scopolamine and hyoscyamine. The antagonist himbacine, from Australian trees of the *Galbulimima* (formerly *Himantandra*) family, recently attracted much attention as a subtype-selective ligand (Anwar-ul *et al.*, 1986). Other examples of natural ligands to muscarinic receptors are the agonists muscarine, from the fly agaric, and pilocarpine, from the South American jaborandi plant (*Pilocarpus jaborandi*). These are all small ($M_r > 500$) molecules. Many of them have found use in medicine.

Later a multitude of synthetic ligands have been developed, for use as pharmaceuticals, research tools or for chemical warfare. Almost any compound with an accessible quaternary

amino group will exhibit some affinity to acetylcholine-binding proteins, and a number of substances developed along these lines, such as alkyltrimethylammonium compounds, carbachol and gallamine, have moderate to high affinity to muscarinic receptors. Many of them do not discriminate between muscarinic and nicotinic receptors and will therefore not be discussed in this treatise.

Some frequently used muscarinic antagonists are N-methylscopolamine (NMS, a derivative of scopolamine), quinuclidinyl benzilate (QNB, a US chemical warfare agent), pirenzepine (a pharmaceutical used in treatment of peptic ulcers) and p-fluoro-hexahydro-siladifenidol. Among the agonists we find oxotremorine (used to induce Parkinson-like symptoms in experimental animals), and the experimental compound McN-A 343.

All the ligands mentioned above bind reversibly to the receptor, but there also are some ligands that bind irreversibly (covalently). They are often used to permanently label or inactivate receptors. One such ligand is N-propylbenzylcholine mustard.

Sometimes it is an advantage if a ligand binds uniformly to all subtypes of receptors, and several such ligands are available. NMS and QNB are examples of such non-selective ligands. But for other purposes, like studying the function of a single subtype of receptor or pharmacological use, selective ligands which bind only or at least preferentially to one subtype are needed. The muscarinic receptors regulate systems as different as heart rate, glandular secretions, and cognitive processes such as memory and learning, and when used as pharmaceuticals, the muscarinic ligands available today often cause unpleasant side effects (Goyal, 1989). This has launched a number of projects attempting to synthesise selective ligands, but despite intense efforts, the results have been disappointing. Pirenzepine, probably the most widely used selective ligand, has a modest five- to six-fold selectivity for m1 over m4 receptors, and the above mentioned "cardiac-specific" antagonist himbacine has proved to have virtually the same affinity to m4 as to m2 receptors (Dörje *et al.*, 1991; Paper V). The supposedly m2-selective agonist oxotremorine has only three times higher affinity for m2 than for m3 receptors (Peralta *et al.*, 1987). Similar poor selectivities are seen with all of the putative subtype-selective ligands, and in general, no truly selective low molecular weight muscarinic ligands are available.

Snakes and their venoms

The mambas

The mambas belong to the snake family called elapids. Some other well-known elapids are coral snakes, cobras and kraits. There are four species of mambas: the Eastern green mamba *Dendroaspis angusticeps*, the Western green mamba (also called Halliwell's mamba) *D. viridis*, Jameson's mamba *D. jamesonii* and the black mamba *D. polylepis*. They are all found in the tropical and southern parts of Africa. The black mamba is largely ground-dwelling, although it is likely to seek refuge in trees when frightened. Reaching lengths of over four metres, it is the

second largest venomous snake in the world (after the king cobra *Ophiophagus hannah*). The other mambas are at most half that size and mainly live in trees. Since they have a potent venom and are very quick in their movements, the mambas have been regarded as highly dangerous. Mortality in cases of black mamba envenomation reaches about 100 %, even when the victims receive hospital treatment (Chapman, 1968). Many exaggerated stories of the black mamba's speed are in circulation. In reality, it should not exceed 16 km/h, but would still be rather difficult to keep up with in brush vegetation. Because of its size, it can strike a person above the waist, and it has been reported to strike so fast that the victim does not at first realise that he has been bitten.

Composition of snake venoms

The venoms of snakes are highly complex mixtures of toxins and other molecules, often acting synergistically. Capillary electrophoresis of black mamba *D. polylepis* venom showed the presence of at least 70 peptides in the range of 6000 and 9000 Da alone (Perkins *et al.*, 1993). This is the mass range where many of the neurotoxins, including the muscarinic toxins, are found. Purification of snake toxins consequently involves considerable work, especially when related toxins differ only by the substitution of one or a few amino acids.

The targets of snake toxins vary. While the elapid venoms have mainly neurotoxic effects, other snake venoms also have significant haemotoxic and myotoxic effects (*i. e.*, effects on blood coagulation and damage to muscle cells, respectively). The venom glands have evolved from salivary glands, and the toxins are in many cases evolved from ancestral digestive enzymes and enzyme inhibitors. Some components of the venom still function as proteases which presumably help the snake to digest its prey. Crotalid (rattlesnake) and viper venoms are particularly rich in proteases, causing severe pain, local necrosis and even liquefaction in the bitten body part. Another enzyme present in most snake venoms is hyaluronidase, an enzyme which degrades the mucopolysaccharides that fill the extracellular spaces of animal tissue. This facilitates the diffusion of toxins into the tissues, and hyaluronidase is therefore sometimes referred to as the spreading factor. Hyaluronidase activity is especially high in viperid and crotalid venoms, while elapid venoms contain less of the enzyme.

The proteases, as well as the phospholipases and cytotoxins discussed below, also influence blood coagulation, although it may not be their main function, since both increased and decreased coagulation can be observed with different components of the same venom. The crotalid and viperid venoms also contain components which specifically influence blood coagulation, like thrombin- like enzymes, prothrombin activator, Factor X activator and platelet aggregation inhibitors, and bites of certain vipers may cause severe internal or cerebral haemorrhage leading to death.

Recently the venoms of the mambas *D. jamesonii* and *D. viridis* were found to contain a potent haemotoxin. It has been named mambin, dendroaspin, and S5C1/SH04 by different research groups (Sutcliffe *et al.*, 1994). It is a platelet-aggregation inhibitor with effects similar

to *Viperidae* haemotoxins, but is structurally related to *Elapidae* neurotoxins such as the mamba muscarinic toxins. This discovery was another example of how the often made distinction between *Viperidae* venoms as being haemotoxic and *Elapidae* venoms being neurotoxic is an oversimplification.

Toxic phospholipases of type A₂ (classification is based on the site of hydrolysis) are present in many snake venoms. It seems that the toxic phospholipases bind to their targets (neurons, muscle fibres, or both) in a specific fashion unrelated to their hydrolytic activity. Some of them exert their toxic effect mainly on nerve cells and others on muscle cells, but a sharp division into neurotoxic and myotoxic phospholipases would be an oversimplification. Damage to the active membranes will allow diffusion of ions across the membranes of nerve cells or muscles, with permanent depolarisation as the result. It is, however, unclear how much of the toxic effect that is related to the hydrolysis of phospholipids in the cell membranes, and whether the specific binding of the phospholipase has any toxic effect in itself. No phospholipases have been detected in mamba venoms.

The cytotoxins (also called cardiotoxins) from various cobra venoms increase the permeability of biological membranes by a mechanism that is poorly understood, but probably requires a specific target protein for toxin binding and results in the formation of a pore in the membrane. The effect is depolarisation and to some extent lysis of muscle cells and a lowered excitability in neurons. The toxins got their alternative name cardiotoxins because of their effect on heart function. Venoms containing cytotoxins usually also contain toxic phospholipases, and the effect of these toxins is strongly synergistic.

The short-chain (60-62 amino acid residues, four disulphide bonds) and long-chain (66-74 residues, five disulphides) α -neurotoxins of kraits, cobras, sea-snakes and some mambas block the nicotinic acetylcholine receptors in skeletal muscle, preventing the opening of the ion channel and thereby the propagation of nerve impulses. α -neurotoxins also have effects on nicotinic transmission in some but not all neuronal systems. On the other hand, the so called κ -neurotoxins (*e. g.*, κ -bungarotoxin and κ -flavitoxin from the kraits *Bungarus multicinctus* and *B. flaviceps*) have high affinity to neuronal nicotinic receptors, but have little effect at the neuromuscular junction (for review, see Chiappinelli, 1991). At least one of the toxic phospholipases, phospholipase A III from the sea snake *Laticauda semifasciata*, binds to the nicotinic receptors and competes for the same binding site as α -bungarotoxin, a long α -neurotoxin from *B. multicinctus* (Harvey and Tamiya, 1980).

Cobra venom also contains large quantities of acetylcholinesterase, which has the effect of lowering the acetylcholine levels at the synaptic cleft and thus has a co-operative effect with the α -neurotoxins in blocking signal transduction.

Mamba venoms are particularly rich in neurotoxins. Some of these are similar to neurotoxins from other snakes. For instance, both long and short α -neurotoxins are found in the venom of *D. viridis*. Surprisingly, α -neurotoxins are almost absent from the venom of *D. angusticeps* (Červeňanský *et al.*, 1991).

Many neurotoxins seem to be unique for the mambas. Among these are the fasciculins, the dendrotoxins, the synergistic-type proteins, two types of calcium channel blockers, and the muscarinic toxins.

Fasciculins are potent non-competitive inhibitors of acetylcholinesterase (for review, see Červeňanský *et al.*, 1991). The effect is similar to that of the common "nerve gases" sarin, tabun and soman in producing elevated levels of acetylcholine at the synapse and the neuromuscular junction. Fasciculins were named after their effect of producing long lasting fasciculations, *i. e.*, uncontrolled muscle twitches, in mice. Other symptoms of fasciculin poisoning are salivation, lachrymation and secretion from the nose (Rodríguez-Ithurralde *et al.*, 1983). Death follows respiratory paralysis at higher doses. Three fasciculins are known, two from *D. angusticeps* venom, and one present in the venoms of both *D. viridis* and *D. polylepis*. They are proteins of 61 amino acids and four disulphides. Acetylcholinesterases from human erythrocytes, rat brain and electric organs of *Electrophorus electricus* and *Torpedo* are inhibited with K_i values in the range 10^{-10} to 10^{-12} M (Červeňanský *et al.*, 1991; Puu and Koch, 1990; Marchot *et al.*, 1993; Radic *et al.*, 1994; Durán *et al.*, 1994). Some other acetylcholinesterases are more than one million times less sensitive, *e. g.*, acetylcholinesterases from chick biventer cervicis muscle and brain, cobra venom and insects (heads of *Musca domestica*, common house-fly), which are inhibited with $K_i > 10 \mu\text{M}$ (Červeňanský *et al.*, 1991; Puu and Koch, 1990). Since the diet of the mambas largely consists of birds, it is rather surprising that the fasciculins should be inactive against avian acetylcholinesterases.

Mamba venom also contains large quantities of acetylcholine, up to 3 % of the dry weight (Welsh, 1967), which has a co-operative effect with the fasciculins in increasing the level of signal substance.

Dendrotoxins selectively block voltage-dependent potassium channels. In neurons, this delays repolarisation of the membrane after an action potential, which causes repetitive or spontaneous firing of nerve signals, with increased and uncontrolled release of neurotransmitters as result (for review, see Harvey and Anderson, 1991). In experimental animals, dendrotoxins produce hypersensitivity to touch and sound, and abnormal behaviour such as moving in circles. Higher doses cause convulsions and death due to massive release of neurotransmitters, such as acetylcholine and glutamate. Dendrotoxins seem to pass the blood-brain barrier of rats and mice at least to some extent, and central effects are seen even when the toxin is injected peripherally, although the lethality is low (LD_{50} about $25 \mu\text{g/g}$ body weight). When injected into the brain, dendrotoxins are several thousand times more lethal (LD_{50} about 5 ng/g body weight).

The so-called synergistic-type proteins consist of two polypeptide chains, which are structurally related to the muscarinic toxins but have only seven cysteine residues each. The two subunits are probably linked together by a single intermolecular disulphide bond, while the other cysteines form intramolecular bonds. Synergistic-type proteins have little effect in themselves when injected into experimental animals, but greatly enhance the lethality of certain

other components of mamba venom when injected simultaneously (for reviews, see Červeňanský *et al.*, 1991; Harvey *et al.*, 1984). Their mode of action is unknown.

Furthermore, mamba venoms contain two unique types of calcium channel blockers. Calcicludine, from *D. angusticeps*, is structurally related to the dendrotoxins (De Weille *et al.*, 1991), while calciseptine (Schweitz *et al.*, 1994) and FS2 (Albrand *et al.*, 1995) from *D. polylepis* are structurally related to the α -neurotoxins. They selectively block certain types of voltage-activated calcium channels.

And here we arrive at the main subject of this thesis: the mamba muscarinic toxins. In contrast to the α -neurotoxins, which block nicotinic receptors and are widely distributed through virtually all elapid and hydrophid (sea snake) venoms, the mamba muscarinic toxins have no known counterpart in the venoms of other snakes. Indeed, no other protein toxins against muscarinic receptors have been established in any species of animals or plants, although some muscarinic activity has been detected in chromatographic fractions of certain jellyfish and other coelenterates (Jolkkonen, M., unpublished results).

The mamba muscarinic toxins are homologous to a large number of snake toxins, including fasciculins, α -neurotoxins, κ -neurotoxins and cytotoxins. At the present, sequences of 175 homologues are known (Mebs and Claus, 1991). The structures have been determined for a number of representatives from the different groups (Table 1 in Albrand *et al.*, 1995), and they all share the same three-dimensional structure, a dense core with three loops protruding outwards, sometimes likened to the image of a hand with the three middle fingers extended. This is the case also with the muscarinic toxins, represented by MT2 (Ségalas *et al.*, 1995a).

The muscarinic toxins do not bind to nicotinic acetylcholine receptors (Adem, A., unpublished results). They are remarkably nonlethal when injected into experimental animals, and no LD₅₀ values are available. In experiments with guinea-pig ileum (Papers II and III) and when injected into the brain of rats (Jerusalinsky *et al.*, 1993), the toxins act as agonists. Since synergistic effects seem to be of great importance in the action of mamba venom (Harvey *et al.*, 1984; Červeňanský *et al.*, 1991), it is likely that the muscarinic toxins will be more potent when injected with the whole venom. Yet it remains to be shown that they contribute significantly to the toxicity of the venom.

Reports from human victims bitten by elapid snakes have described a feeling of passivity, equanimity and resignation, even disinterest in the outcome (Kauffeld, 1963). It is known that many cholinergic agents have an incapacitating effect on the cognitive functions at doses where they do not make the subject physically unable to move about, and although it can be questioned whether cholinergic snake toxins in general have such behavioural effects, this is the case at least with dendrotoxin (for review, see Harvey and Anderson, 1991). Snake venoms are often assumed to have the sole function of paralysing or killing the prey. It might be worthwhile to consider the possibility that some components of snake venoms also interfere with the normal behaviour of the prey, by confusing the victim or blocking its normal responses to an attack. A serious objection to such a theory, at least as far as mammals are

concerned, is the question of how peptide toxins would cross the blood-brain barrier to any larger extent.

SCOPE OF THE PRESENT WORK

In the late 1980s, it was known that mamba venom contains toxins which interact with muscarinic receptors (Adem *et al.*, 1988). These were soon found to be unique in several ways: They are the only known animal toxins specific for muscarinic receptors, the only known muscarinic ligands of peptide nature, and the only known muscarinic ligands with a high degree of subtype specificity. This obviously merited them for a deeper study, and I decided to abandon a previous research project in order to concentrate on the mamba muscarinic toxins. These were soon found to compose a rather large family of closely related muscarinic toxins, and in order to limit the scope of the work, two species of mambas were chosen for study, the Western green mamba *D. angusticeps* and the black mamba *D. polylepis*. In the following discussion of the papers that this thesis is based upon, they will simply be called green mamba and black mamba, respectively.

When I started on this project I had little experience in the study of ligand-receptor interactions, and so I originally set out only to isolate a number of representatives for this new and fascinating class of snake toxins, to determine their amino acid sequences, and to investigate their structural relations to each other and to other types of snake toxins. This thesis describes the isolation of eight of the twelve known mamba muscarinic toxins, and the sequence determination of seven of them. The partial sequence of an eight toxin is also given.

Eventually, it became clear that we needed to perform our own binding studies in order to work efficiently, and after I had received some training at the Institute of Chemical Physics at Tartu University, the determination of toxin affinities became a major part of the work. However, the subtype selectivity of the toxins made interpretations of results obtained with heterogeneous receptor populations difficult, and much of the data produced in this way still remains unpublished. After a co-operation with the Marion Merrell Dow Research Institute in Strasbourg had been established, we decided to perform a thorough screening of the affinities of all major muscarinic toxins to individual receptor subtypes cloned into Chinese hamster ovary (CHO) cells. A compilation of the results is presented for the first time in this thesis.

We also investigated the binding kinetics of the toxins, although we limited these studies to three toxins. The results with two of them are reported in this work.

Some studies on the pharmacological effects of the toxins were done, but unfortunately only a small part of the planned project could be carried out before it was postponed due to difficulties with the cell cultures involved. Some preliminary experiments on the pharmacology of the toxins are described in the present work.

In the past, we have often stressed the great potential of the muscarinic toxins in receptor research, and quite recently this has been demonstrated by the autoradiographic mapping of m1

muscarinic receptors in different regions of rat brain. Although the manuscript (Paper IX) is not yet in its final form, it is with great pleasure that I include this example of the practical value of mamba muscarinic toxins in my thesis.

INTRODUCTION TO THE METHODS USED

As mentioned above, the scope of the present work is the characterisation of the structure and function of the muscarinic toxins. For this purpose, three things are needed: purified muscarinic toxin, a source of muscarinic receptors, and a method of observing and measuring the binding of toxin to the receptor. These subjects will be addressed below. The protein chemical methods involved in the structure determination, being common to many areas of biochemistry, are described in the individual papers.

Isolation of muscarinic toxins: Separation strategy

The aim has been to in the first steps achieve a group separation of the muscarinic toxins, in other words, to produce a fraction containing all of the muscarinic toxins and with most of the other components removed. Since all the known muscarinic toxins are of similar size (M_r 7000 – 7600), gel filtration is suitable as a first step in fractionation of the venom. In the second step, cation exchange on Bio-Rex 70 was performed under conditions where the muscarinic toxins passed unretarded through the column, while the majority of other components was retarded. With these two steps, a fraction highly enriched in muscarinic toxins was obtained.

This fraction was then further separated into its individual components using cation exchange and reversed-phase HPLC. These steps differ slightly for separation of green and black mamba toxins, and are described in detail in Papers IV and VI, respectively. The peaks were screened for muscarinic activity by measuring the decrease in the binding of a radiolabelled muscarinic antagonist, such as [3 H]-NMS or [3 H]-QNB.

An alternative method for detecting muscarinic toxins is amino acid analysis, which has the advantage that it also provides an identification of the toxin. Because of the high level of homology between the muscarinic toxins, it is possible to fairly accurately predict from the composition if a molecule is a muscarinic toxin or not. Naturally, this approach will never lead to the discovery of structurally different muscarinic toxins.

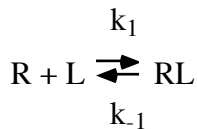
We have found it advantageous to increase the scale of the preparations, using up to ten grams of freeze-dried venom as starting material. It seems that several of the muscarinic toxins are present in the venom only in trace amounts and cannot otherwise be isolated in quantities sufficient for analysis. It is not unlikely that the number of known muscarinic toxins could be doubled by careful examination of every minor peak in the chromatograms.

Binding studies

Basic concepts in ligand binding

The binding of ligand to receptor in many ways resembles the binding of substrate to enzyme, and the terminologies and mathematics are very similar. A few general aspects of ligand binding to receptor will be presented here, although the corresponding concepts for enzyme kinetics may be studied in most textbooks of biochemistry.

The simplest case of binding of a ligand to a receptor is where one ligand molecule (L) binds reversibly to one receptor molecule (R) in a single step, characterised by the rate constants k_1 and k_{-1} for the association and dissociation, respectively:

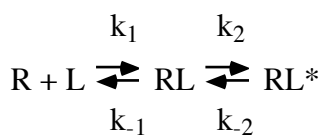


The affinity of the ligand for the receptor is then characterised by the dissociation coefficient K_d , usually defined as

$$K_d = [\text{R}][\text{L}] / [\text{RL}],$$

where $[\text{R}]$, $[\text{L}]$ and $[\text{RL}]$ are the concentrations at equilibrium of R, L and the receptor-ligand complex RL, respectively. K_d is related to the rate constants as $K_d = k_{-1} / k_1$.

It is rather common that the formation of the ligand-receptor complex proceeds in two or more steps, where first a "loose" complex forms and dissociates at a fast rate, but can transform, or isomerise, into a "tight" complex RL^* with significantly lower dissociation rate. What constitutes a discrete step in the binding is a question of definition, and will in practice be determined by the sensitivity of the methods of observation. It is often practical to limit the distinction to a fast initial step and a slower isomerisation step :



The loose complex RL may be too short-lived to observe with standard methods. What is observed is then the formation of the tight complex RL^* , with the apparent dissociation constant $K_d' = K_1 \cdot K_2 = (k_{-1}/k_1) \cdot (k_2/k_2)$.

The binding is also characterised by a parameter called the Hill coefficient, which (in the case of a homogenous receptor population) describes the degree of co-operativity between binding sites. A Hill coefficient that significantly differs from unity indicates that the ligand's affinity changes with receptor occupancy, which can be explained with allosteric interactions between multiple binding sites. The often drawn reverse conclusion, that a Hill coefficient of one proves that the receptor has a single binding site for the ligand, is however not quite valid. What can be said in this case is merely that the binding sites are independent of each other.

When two different ligands L and I (for inhibitor) bind to the same receptor, three different cases are possible.

In the first case, called competitive inhibition, the binding of ligand and inhibitor to the receptor are mutually exclusive and no ternary complex RLI can be formed.

In the second case, called uncompetitive inhibition, the inhibitor can bind to ligand-receptor complex but not to receptor alone; RL and RLI but not RI can form. Uncompetitive inhibition is most common with receptors (or enzymes) that bind two or several ligands (substrates).

In the third case, called non-competitive inhibition, ligand and inhibitor may bind separately or together to the receptor; the complexes RL, RI and RLI may form. In order to be a case of inhibition, L must have lower affinity for RI than for R. In enzyme kinetics, the term "non-competitive inhibition" is often used for the special case where the substrate L has similar affinities to R and to RI, but the complex RLI is enzymatically less active. Such a definition would be meaningless when measuring ligand binding to a receptor, and in this case, the terminologies are not interchangeable between enzyme kinetics and ligand binding. Furthermore, while in the ideal case of enzymatic "pure non-competitive binding" the substrate has exactly the same affinity for R (enzyme) as for RI (enzyme-inhibitor complex), these affinities are likely to differ in the real case. This is often referred to as "mixed-type inhibition". That term might be more suitable than "non-competitive inhibition" for use in the context of ligand binding.

At a fixed concentration of the ligand L, it is possible to determine the concentration of inhibitor that decreases the binding of L to receptor by 50%. This concentration is called IC₅₀ (for Inhibitory Concentration, 50 %). If the K_d and concentration of the ligand L are known, it is possible to calculate the inhibition constant K_i for the inhibitor.

The relation between K_i and IC₅₀ depends on the type of inhibition. For competitive inhibition, it is $K_i = IC_{50} / (1 + [L] / K_d)$ and K_i equals the dissociation constant K_d of the inhibitor. This relation is known as the Cheng-Prusoff formula (Cheng and Prusoff, 1973). In practice, it has become rather common to use the Cheng-Prusoff formula even in the absence of information on the type of inhibition. In this case, the reported K_i value should be regarded as a transform of the IC₅₀ value rather than as a true dissociation constant.

Measuring the binding of toxins

The binding of a native toxin to membrane-bound receptors is not directly observable. One solution to this problem is to label the toxins with a radioisotope. Most commonly used is ¹²⁵I, which can be made to react with the tyrosine residues of the toxins. The toxin is then incubated with the receptors, bound toxin is separated from the toxin remaining in solution, usually by filtration or centrifugation, and the bound radioactivity is measured. The method involves several difficulties.

Firstly, the iodination may render the toxin inactive, either by modifying a Tyr residue

crucial for toxin binding or because the oxidising conditions during iodination damage the toxin in some other way, like oxidation of disulphides. The affinity of the toxin may also change in more a more subtle way, remaining the same for one receptor subtype but decreasing for another. Both these cases will be exemplified in this work.

Secondly, there is often a problem with non-specific binding of the radiolabelled toxin. Many proteins behave in a "sticky" way, adsorbing to lipid membranes, to membrane proteins, and to surfaces on the experimental equipment (test tubes, filters and so forth). The radiolabelled muscarinic toxins act in this way. While the number of specific binding sites (receptors) is limited by the receptor density, the number of non-specific binding sites is practically infinite. As a consequence, the non-specific binding increases linearly with toxin concentration while specific binding asymptotically approaches a limiting value, and the non-specific binding must be determined for each toxin concentration used and subtracted from the observed, total binding. At higher toxin concentrations the non-specific binding may completely mask the specific binding, making any observation impossible.

Thirdly, the method is only practical with purified toxins. Measurements with an iodinated mixture of peptides, such as a fraction from gel filtration, could be expected to give extremely high non-specific binding masking any specific binding of one component in the mixture. Radioiodination of peptides is also too expensive and cumbersome to be of any real use in routine screening of, for instance, chromatographic fractions.

Still, for certain purposes, this is the method of choice. It may reduce the potential for errors, since the binding of toxin is observed more directly and no assumptions need to be made regarding the reporter ligand's affinity and the type of inhibition the toxin exerts on its binding. The one assumption that is usually made instead, namely that the radiolabelled toxin has the same affinity as the native toxin, can and should be checked by comparing results obtained with different methods.

Another use for radiolabelled toxins is autoradiography, where the toxin is incubated with, for instance, slices of tissue and the binding is detected by photographic or scintillation techniques. The method produces a picture, where the toxin binding to various regions is directly visible. It can show differences in receptor density with high resolution and visualise patterns in receptor distribution which would be difficult to detect with other methods.

An alternative method of measuring toxin binding is to use a radioligand ("reporter ligand"), which competes with the toxin for its binding sites. In this case, the toxin will be an inhibitor of the radioligand binding, and if the affinity of the reporter ligand and the type of inhibition is known, the affinity of the toxin can be calculated as described earlier.

The practical difficulties with this method are usually insignificant. If the reporter ligand is a small molecule, its non-specific binding will probably be very low, and will in any case be independent of the toxin concentration. The toxin will have its native binding properties, and whole venom and crude fractions can be analysed - with the exception of preparations containing enzymes which degrade the membranes or the receptors, or other membrane-

damaging (cytolytic) substances. That can be the case with, for instance, snake venoms containing phospholipases and toxic secretions from certain marine organisms.

With both these methods, it is most common that the toxin binding is studied at equilibrium, especially when the aim is only to determine the affinity of the toxin to the receptor. In this case the toxin (and the reporter ligand when used) is incubated with the receptors for a standard time, after which the binding is assumed to have reached completion, before any measurements are made. It is, however, often rewarding to study the process of toxin binding, for instance by drawing and analysing aliquots of the incubation mixture at regular time intervals. This allows the determination of rate constants and can give deeper insight into the mechanism of toxin binding. Some examples of this are given in this thesis.

A rather different method for measuring toxin binding is by using more complex biological systems and observing the effects upon addition of toxin. These may be measured mechanically (*e. g.*, muscle contraction in gut), spectroscopically (*e. g.*, calcium influx into cells treated with calcium-binding fluorescent dye), radiologically (*e. g.*, binding of a radioactive GTP analogue to G-protein) or electrically (*e. g.*, patch-clamp technique). The effects of toxin may also include the inhibition of the response to a given external stimulus. Because of the complexity of the systems studied, elaborate control experiments may be necessary. For example, a tested substance may produce contraction in smooth muscle, and the effect is shown to be reversed upon addition of atropine. This may be incorrectly interpreted as proof that the substance is a muscarinic agonist, when it is possible that it instead acts on calcium channels, initiating acetylcholine release, or an anticholinesterase, inhibiting the breakdown of spontaneously released acetylcholine. It might even be a selective muscarinic antagonist, blocking the inhibitory presynaptic muscarinic receptors.

Another property of these so called pharmacological assays is that in contrast to the simple binding assays described earlier, which typically yield results in physical standard units, the results are often obtained in a system of units specific for the method used and cannot directly be translated to a more general scale of measurement. However, when applied competently, the pharmacological assays can give insights impossible to achieve with simple binding assays.

Sources of muscarinic receptors

Several of the mamba muscarinic toxins bind preferentially to a limited number of subtypes, while exhibiting almost no detectable binding to others. When screening a sample for muscarinic activity, it is therefore practical to test it on cell membranes expressing a mixture of receptors. The brain expresses relatively high concentrations of receptors m1 to m4 (Table 2) and is a suitable source for preparation of such membranes. (The expression of m5 receptors is very low in all tissues). When brain tissue is homogenised, the synaptic nerve endings become "pinched off" and form vesicles rich in muscarinic receptors, called synaptosomes. These can be prepared in a crude form as the fraction sedimenting at a centrifugation speed above 10 000 g

but not at 1000 g, or in a purer form by equilibrium centrifugation in a density gradient.

A common source of synaptosomes is rat brain. The rats are decapitated in a guillotine especially devised for this purpose, the cranium is opened with pliers, and the brain can be lifted out with a spatula. Because of the size of the brain, several specimens are usually needed for a series of experiments. As laboratory rats are docile and curious animals, it is easy to introduce the first rat into the guillotine, but since the following ones often will be frightened by the smell of blood, the decapitation can evolve into a rather messy procedure.

An alternative source is porcine brain. The homology of porcine receptors to human is 99 % for m1, 97 % for m2 and 96 % for m3 (Peralta *et al.*, 1987; Akiba *et al.*, 1988), and the homology to rat receptors is 98% for m1, 95 % for m2 and 94 % for m3 (Brann *et al.*, 1987; Gocayne *et al.*, 1987; Akiba *et al.*, 1988). The use of pig brain has the advantages that fresh material is readily available in large quantities at negligible cost, that the membranes from a single preparation, kept frozen in aliquots at -70 °C, constitute a long-lasting stock with identical receptor density and distribution, and that it does not involve the unpleasant and time-consuming work of killing and dissecting laboratory animals.

When the binding constants of a toxin are to be determined, results obtained with mixtures of receptor subtypes can be difficult to interpret. If the toxin has similar affinity to all subtypes the case is very simple. If it binds to one subtype with high affinity and to all others with far lower affinity, it is also possible to observe the high-affinity binding separately and determine the binding constants. But when the affinities for two subtypes differ by about an order of magnitude, the binding curves will overlap and can be difficult to resolve from each other. It is generally an advantage if one can use single subtypes of receptors and determine the affinity to each subtype separately. In the case of m2 receptors, it is possible to use rat or porcine heart muscle, which is known to express only subtype m2 (Li *et al.*, 1991; Mayanil *et al.*, 1991). Homogenous populations of other subtypes are not readily available in natural materials. Some experimenters have used specific regions from brain, known to be enriched in certain subtypes, as their source of receptors. By skilful comparison of results obtained with a number of such preparations, it is possible to draw some conclusions regarding the affinity to the various subtypes. Unfortunately, the reports on what subtypes are expressed are sometimes contradictory, and the reports often use the pharmacological (M1, M2a, M3 and so forth) rather than the molecular (m1 – m5) characterisation (see Table 1). Furthermore, the dissection of the appropriate brain regions requires surgical and anatomical skills beyond the average biochemist's. It should also be kept in mind that synaptosomes and other cell membranes contain other receptors than the muscarinic, and that these also may bind the ligands used.

Today it is often easier to use cloned receptors, using cells that in themselves express no or very few receptors, but have been genetically manipulated to express the desired receptor subtype. These cells may be homogenised and a membrane preparation produced much in the same way as described above, or they may be used directly from culture to study the pharmacological effects of the toxins.

DISCUSSION OF THE INDIVIDUAL PAPERS

Characterisation of the first muscarinic toxins: Papers I, II and III .

The toxin MT2 is the most abundant in the green mamba venom, and so it was logical to begin our studies with MT2. The amino acid composition suggested that the muscarinic toxins were structurally related to the α -neurotoxins and cytotoxins. Sequence determination confirmed that this was the case (Paper I).

While the sequence determination of MT2 was rather straightforward, the sequencing of MT1 (Paper II) was considerably more problematic. During the work I learned that, contrarily to the popular opinion, derivatisation of cysteine with 4-vinylpyridine often reduces the solubility of peptides. The matter was further complicated by the fact that the middle part of the MT1 sequence is highly enriched in hydrophobic residues. The methods and precautions developed in order to solve these problems, like the use of acidic or organic solvents in gel filtration of the peptide fragments, were thereafter always used in sequence determination of muscarinic toxins.

Meanwhile, we had started to investigate the peculiar binding of the muscarinic toxins, which produced only about 50 % inhibition of the binding of radiolabelled "reporter ligands" such as [³H]-QNB or [³H]-NMS. Unfortunately, binding studies with MT2 proved to be difficult to perform. Repeated attempts at radiolabelling MT2 with different methods consistently yielded an inactive toxin, and binding studies with unlabelled MT2 gave rather poor reproducibility. As we now know, MT2 is remarkably unstable and easily becomes inactivated on freezing and during storage, a property it may share with the black mamba toxin MT β (Paper VI). The reasons for this instability are not clear, but may be due to the presence of an exceptionally unstable Asp-Pro bond in the third loop of MT2 (Ségalas *et al.*, 1995b). Other muscarinic toxins are much more stable; for instance, MT1 retains full activity even after heating to 95 °C (Jolkkonen, M., unpublished results).

We redirected our efforts and found that the binding of MT1 was highly reproducible and seemed to retain all of its activity after iodination. Binding studies with [³H]-NMS as reporter ligand (Paper II) supported the theory that the toxin had different affinities for different receptor subtypes. Binding of MT1 to membranes from porcine heart (m2 receptors) was observed only at very high toxin concentrations, with a K_i in the micromolar range (Paper II, Fig. 1). The binding to synaptosomal membranes was more complex. Significant binding took place at lower toxin concentrations (1-100 nM), but due to overlapping with the binding to m2 receptors, the IC₅₀ of the first binding step could not be accurately determined. In order to resolve the two phases from each other, I repeated the experiment using a different reporter ligand, AF DX-384, which has higher affinity to m2 than to other receptors. Thus the second step, inhibition of radioligand binding to m2 receptors, was shifted to higher toxin concentrations relative to the first step, and a plateau could be observed between the two sigmoid segments of the binding curve. In the first binding step, 37 % of the radioligand

Fig. 1. Amino acid sequences of mamba muscarinic toxins.

The sequence of MT5 was assembled from the sequences 1–36 (direct sequencing of the pyridyl ethyl derivative) and the chymotrypsin fragments 37–52 and 53–65. The segments were ordered by homology to other muscarinic toxins. C-terminal determination by hydrazinolysis gave glutamic acid in a yield of 96% and no traces of other amino acids. The sequence agrees with the amino acid composition and mass spectrometry data.

MT7 has been sequenced only to amino acid 40. The sequence 41–65 of MT7 has been constructed from amino acid content and homology with other toxins, and thus it does not based on solid experimental data. C-terminal determination by hydrazinolysis gave lysine in a yield of 84% and no traces of other amino acids.

The *D. polylepis* peptide CM-3 (Joubert, 1985) is included because of its close homology with MTβ. Its affinities to muscarinic receptors has not been assayed.

The sequences of 29.6 = DPD 29.6 and 10.3 = DP II 10.3 were determined from a cDNA library of *D. polylepis* venom components (Smith, L. A., personal communication). The corresponding peptides have not been isolated. The sequences are included because of their homology with the muscarinic toxins. DPD 29.6 differs from MTα only in positions 40, which is Asn in DPD 29.6 and Trp in MTα, and 47, which is Ser in DPD 29.6 and Pro in all other sequences. The sequence of DP II 10.3 makes a "chimerical" impression: the N-terminal part of DP II 10.3 is similar to MT3 (residues 1–40, five differences) while its C-terminal part is more similar to MTα (residues 41–66, three differences). m1 = m1-toxin from *D. angusticeps* (Max *et al.*, 1993a).

Multiple sequence alignment was made with the CLUSTAL W (1.5) program (Thompson *et al.*, 1994), using the neighbour joining method (Saitou and Nei, 1987).

The following parameters were used:

Gap Opening Penalty: 10.00 Gap Extension Penalty: 0.05
 Delay divergent sequences: 40 % Protein weight matrix: BLOSUM series
 Use negative matrix: OFF

The resulting alignment score was 22381.

CLUSTAL W (1.5) is available by anonymous ftp from the EMBL server at <ftp.embl-heidelberg.de/pub/software>.

Invariant amino acids are marked with two dots, favoured substitutions with one dot.

	1	10	20	30	40	50	60	66		
MT1	LTCVTSKSI	FGITTEN	CPDQNL	CFKKWY	YIVPRYS	DITWGCA	ATCPKPT	NVRETIR	CCETDKC	NE
	:::	·:	·:	::	:::	::	·	·	·	·
MT2	LTCVTTK	SIGGVTT	EDCPAGQ	NVCFKR	WHYVTP	KNYDII	KGCAAT	CPKVDNN	—DPIRCC	GGTDC
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
MT3	LTCVTKN	TIFGITT	ENCPAGQ	NLCFKR	WHYVIP	RYTEIT	RGCAAT	CPPIPEN	Y—DSIH	CCKTD
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
MT4	LTCVTSKSI	FGITTEN	CPDQNL	CFKKWY	YIVPRYS	DITWGCA	ATCPKPT	NVRETI	HCCE	TDKCNE
	:::	·:	·:	::	:::	::	·	·	·	·
MT5	LTCVTSKSI	FGITTED	CPDQNL	CFKRRH	YVVPKI	YDITRGC	VATCPK	PENY—	DSIH	CCKTD
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
MT7	LTCVKNS	NIWFPT	SEDCPD	QNLCFK	RWQYIS	PRMYDF	TRGCAAT	CPKAEYR	—DVIN	CCGTD
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
m1	LTCVKNS	NIWFPT	SEDCPD	QNLCFK	RHWYIS	PRMYDF	TRGCAAT	CPKAEYR	—DVIN	CCGTD
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
MTα	LTCVTSKSI	FGITTEN	CPDQNL	CFKKWY	YLNHRYS	DITWGCA	ATCPKPT	NVRETI	HCCE	TDKCNE
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
MTβ	LTCVTSKSI	FGITTED	CPDQNL	CFKRRH	YVVPKI	YDITRGC	VATCPI	PENY—	DSIH	CCKTD
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
CM-3	LTCVTSKSI	FGITTED	CPDQNL	CFKRRH	YVVPKI	YDITRGC	VATCPI	PENY—	DSIH	CCKTE
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
10.3	LTCVTKD	TIFGIT	TQNCPA	QNLCFI	RQHYV	NHRYTE	ITRGC	AATCPK	PLNVRE	TIHCC
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:
29.6	LTCVTSKSI	FGITTEN	CPDQNL	CFKKWY	YLNHRYS	DITNGCA	ATCSKPT	NVRETI	HCCE	TDKCNE
	:::	·:	·:	·:	·:	·:	·:	·:	·:	·:

binding was inhibited. The second (low-affinity) binding step corresponded to m2 (heart) receptors (Paper II, Fig. 1), and possibly also other subtypes. According to available sources on the proportions of the different receptor subtypes in brain, the subtype comprising about 37 % of the total number of receptors, not being m2, would be either m1 or m4. Although this holds true, the ratios of receptors quoted in Paper II were incorrectly translated from the data in Table 3 of Waelbroeck *et al.*, 1990. See Table 2 for a compilation of reported subtype ratios in rat cortex, including the data of Waelbroeck *et al.* in its correct form.

It was now necessary to determine which of these two subtypes that MT1 bound to in the first step. Knowing the affinities of AF DX-384 to the receptor in question, we would be able to calculate the affinity of native MT1 to the receptor.

A sample of MT1, radiolabelled with ^{125}I , had been prepared and found to bind to synaptosomal membranes with a K_d of 20 nM in the high-affinity binding step (Paper II). Synaptosomal membranes were incubated with a low (2.5 nM) concentration of the radiolabelled toxin, ensuring that the toxin would bind only to its high-affinity site, and pirenzepine at varying concentrations. Since pirenzepine has about six times higher affinity to m1 than to m4 receptors, and knowing the K_d of $[^{125}\text{I}]\text{-MT1}$, it would be possible to deduce which subtype $[^{125}\text{I}]\text{-MT1}$ and pirenzepine were competing for. The binding of $[^{125}\text{I}]\text{-MT1}$ was inhibited to 80 % with a K_i of 6.5 nM, which was somewhat lower than the reported affinity of pirenzepine for m1 receptors, and far lower than for other subtypes. From this we concluded that the high-affinity binding of MT1 was to m1 receptors (Paper II).

Table 2. Relative density of receptor subtypes m1–m5 in rat cortex, as reported by various research groups.

Reference	m1	m2	m3	m4	m5	Method
Wall <i>et al.</i> , 1991a	34%					Immunological
Wall <i>et al.</i> , 1991b			10%			Immunological
Levey <i>et al.</i> , 1991	40%	37%	0%	15%	0%	Immunological
Yasuda <i>et al.</i> , 1993				24%	0.3%	Immunological
Li <i>et al.</i> , 1991		20%				Immunological
Waelbroeck <i>et al.</i> , 1990	34%	22%	10%	34%		Ligand binding

Using the K_d of AF DX-384 for m1 receptors, the affinity of MT1 to m1 receptors was calculated from the IC_{50} value from competition with $[^3\text{H}]\text{-AF DX-384}$. This gave a K_i of 32 nM, to be compared with the K_d of 20 nM for radiolabelled MT1. Thus the toxin retained full activity after iodination.

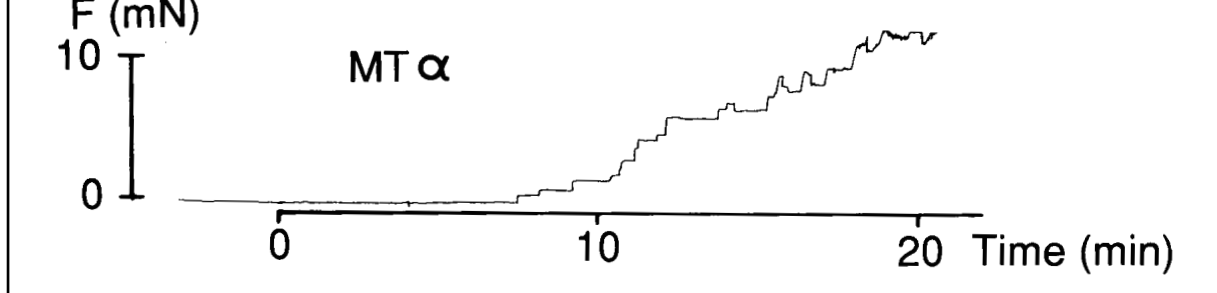
Similar binding curves were made with several muscarinic toxins, using [³H]-NMS as reporter ligand and porcine heart and brain membranes as source of receptors. The black mamba toxins exhibited less evidence of subtype selectivity. For instance, the black mamba toxin MT β started to inhibit the binding of [³H]-NMS to heart and brain receptors at the same concentration, about 100 nM (Paper III). The binding to brain receptors appeared to be monophasic, and although practical limitations did not allow sufficiently high toxin concentrations to reach zero radioligand binding, extrapolation of the inhibition curve suggested that the lower plateau would be near zero. This is in accordance with what would be expected of a non-selective ligand. As there was no point in trying to resolve the curve into a biphasic one, no experiments with subtype-selective ligands or radiolabelled toxin were made.

The statement in Paper III that the black mamba toxins are quite stable could be in error; as already mentioned, some preparations of MT β may have been partially inactivated.

With the fact that MT1 is subtype-selective finally established, we addressed the question of whether the toxin is an agonist or an antagonist. We decided to make a simple experiment, using guinea-pig ileum (a part of the small intestine) as test system. A complication with ileum is that it is a complex living system, with diverse and sometimes incompletely known pathways leading to the same observable effect, contraction of the smooth muscle in the ileum, which is measured by a simple electromechanical transducer and transferred to graph paper. Many substances of different nature can produce a contraction, but if the effect is blocked by atropine, then muscarinic receptors are involved at least in some step of the pathway. The simplest explanation is that the substance is a muscarinic agonist; the alternative explanation that the substance facilitates release of acetylcholine at the motor endplate. The effects of a muscarinic antagonist can be observed by simultaneously adding muscarine to the test solution, in which case the antagonist inhibits the contraction.

It was found that at a concentration of 670 nM (5 μ g/ml), MT1 (Paper II and III) and the black mamba muscarinic toxins MT α (Jolkkonen, M., unpublished results) and MT β (Paper III) all produced contraction in ileum that had been pre-treated ("stripped") to facilitate access to the nerve endings. The onset of the effect was immediate with MT1, although the contraction took about ten minutes to reach its maximum (Paper II, Fig. 4). The effect persisted for over 30 min. In the case of MT β , the first effects appeared after ten minutes, increased over the next ten minutes, and then decreased over ten minutes until the ileum was again fully relaxed (Paper III, Fig. 4). The effect of MT α , tested under identical conditions, was similar to that of MT β in the slow onset, and to MT1 in that the effect persisted for over 30 min (Jolkkonen, M., unpublished results; Fig. 2 of the present work). With all three toxins, the effect was immediately reversed by atropine. None of the toxins had any effect on untreated ileum. The results suggest that they are all muscarinic agonists, with the reservations made above.

Fig. 2. Effect of MT α on stripped guinea-pig ileum. The experimental procedure was identical to that described in Paper III.



The first isotoxin: Paper IV.

We had long been mystified by the appearance of traces of histidine in our preparations of MT1. It was difficult to believe that it was due to contamination with another peptide, since all other amino acids gave good integer values in analysis. For some time we were satisfied that repeated chromatography removed these traces. Finally, I isolated the mysterious contaminant and found that it had the same composition as MT1, with the exception that one arginine was replaced with histidine. Proteolytic degradation of the new toxin, called MT4, followed by amino acid analysis of the fragments, indicated that the substitution was in position 57. Determination of the amino acid sequence confirmed that this was the only difference in the sequence (Paper IV). It would later be found that many toxins exist which differ only in one or a few amino acid substitutions, and that these could give considerable insight into the binding of the toxins. These groups of closely related toxins were called isotoxins. In Paper IV, the isolation of seven muscarinic toxins (MT1–MT7) was described, although only MT4 was characterised. The amino acid compositions of the toxins which have not yet been fully sequenced are presented in Table 3 of this thesis. The affinities of MT1 and MT4 are discussed in the following section.

Binding studies using cloned receptors : Papers V and VI.

As pointed out earlier, the methods used so far for determining the subtype selectivity of the toxins had serious limitations. In animal tissues, only m2 receptors could be found as a homogenous population.

Natural raw materials being excluded, we tried some systems with cloned receptors. In co-operation with the Marion Merrel Dow Research Institute in Strasbourg, we successfully determined the affinities of several muscarinic toxins for m1 – m5 receptors expressed in Chinese hamster ovary (CHO) cells. The experimental procedures are described in Paper V. The results have been published only for a few of the toxins (Papers V and VI), and additional data is published for the first time in this thesis.

Table 3. Amino acid compositions of toxins not yet sequenced. The isolation is described in Paper IV (*D. angusticeps* toxins MT6 and MT7) and Paper VI (*D. polylepis* toxin MT γ). Where glycine content is uncertain, the lower figure is likely to be correct. Additional data can be found in Appendix 1.

Residue	MT6	MT7	MT γ
Asx	8	9	7
Thr	9	5	8
Ser	1	4	3
Glx	4	4	5
Pro	4	4	4
Gly	4	3 (4?)	3 (4?)
Ala	2	3	2
Cys	8	8	8
Val	3	2	3
Met	0	1	0
Ile	6	3	4
Leu	2	2	2
Tyr	3	3	3
Phe	2	3	2
His	2	0	1
Lys	3	5	5
Arg	3	4	3
Trp	1	2	2
Residues	65	65	65

The results showed that subtype selectivity is quite common in muscarinic toxins. One toxin seemed especially promising for further research: the green mamba muscarinic toxin MT3. It was found to have high affinity ($K_i = 2.0$ nM) to m4 receptors, 40 times lower affinity ($K_i = 78$ nM) to m1 receptors, and virtually no affinity to other subtypes (Paper V). Its sequence was determined (Paper V) without any of the difficulties encountered with MT1, using the tryptophan-specific reagent BNPS-skatole, an excellent tool for peptide cleavage which still has found surprisingly little popularity. The sequence of MT3 does not show very close similarity to any other muscarinic toxins. The toxin seems to be quite stable and is by far the most selective ligand known for m4 receptors, which should make it very valuable in receptor research.

Although far less work has been done on muscarinic toxins from the black mamba than on those from the green mamba, three muscarinic toxins, called MT α , MT β and MT γ , have

been isolated from its venom, and the first two of them have been sequenced (Paper VI). Very little work has been done on MT γ due to the small amounts of toxin isolated.

The mamba venoms contain a large number of muscarinic toxins, of which some are present in very low amounts. Several of the toxins which have so far not been published upon are such minor components which can be isolated in amounts sufficient for analysis only by increasing the scale of the preparation, using up to ten grams of venom as starting material, and probably they have often been discarded as contaminants.

It is unclear why the mambas produce such a wide array of muscarinic toxins, and the simplest explanation would be to consider it the effect of random genetic drift. With the sequencing of MT α and MT β , this theory became less plausible. Although the black and the green mamba are physically quite dissimilar and must have diverged from their common ancestor considerable time ago, the amino acid sequences of their toxins have been strongly conserved. MT α differs from MT4 only by three residues, and MT β differs from MT5 by a single residue (Paper VI and Jolkkonen, M., unpublished results; Fig. 2 of the present work). These differences between the species are much smaller than the differences within a species (Fig. 3); for instance MT1 and MT2 differ in 23 positions, MT1 and MT3 in 19 positions, and MT2 and MT3 in 22 positions. The different muscarinic toxins must therefore be assumed to have remained fairly unaltered since the species diverged, and an alternative explanation to the diversity among them must be found. One such theory can be based on the fact that although the acetylcholine-binding, transmembrane part of the receptor is fairly similar in all subtypes, the extramembraneous loops are quite dissimilar. Consequently the toxins, being large and bulky molecules compared with the less selective low molecular weight ligands, might not fit well to more than one subtype of muscarinic receptors, necessitating the production of several toxins in order to achieve binding to all subtypes of receptors.

Again, things were not that simple. The black mamba toxin MT α proved to have high affinity (K_i ranging from 3 to 44 nM) to all subtypes (Paper VI). The question remains unanswered what evolutionary pressure has made the mambas produce a large number of highly selective muscarinic toxins, when a single toxin exists which, as it seems, could replace the rest of them.

The low subtype selectivity of MT α was remarkable in another way as well. It was the first example of how the isotoxins, of which we at present know three groups (MT1 – MT4 – MT α , MT5 – MT β – CM3, MT7 – m1-toxin) (Fig. 3), sometimes have very different selectivity. In the cases where the amino acid sequences differ only in one small region, it has allowed us to draw some conclusions regarding the receptor-binding sites of the toxins. MT α differs from MT4 only in the region 31–33, which is Leu-Asn-His in MT α and Ile-Val-Pro in MT4 (Paper VI). This region, in the toxin's second loop (Paper VI, Fig. 4), obviously takes part in the binding, since the substitutions change the selectivity from that of MT α (high affinity to all subtypes) to that of MT4 (high affinity only to m1 and m4 receptors, while affinities to the other three subtypes is at least 200 times lower) (Paper VI).

Fig. 3. Phylogenetic tree of mamba muscarinic toxins and closely related sequences.

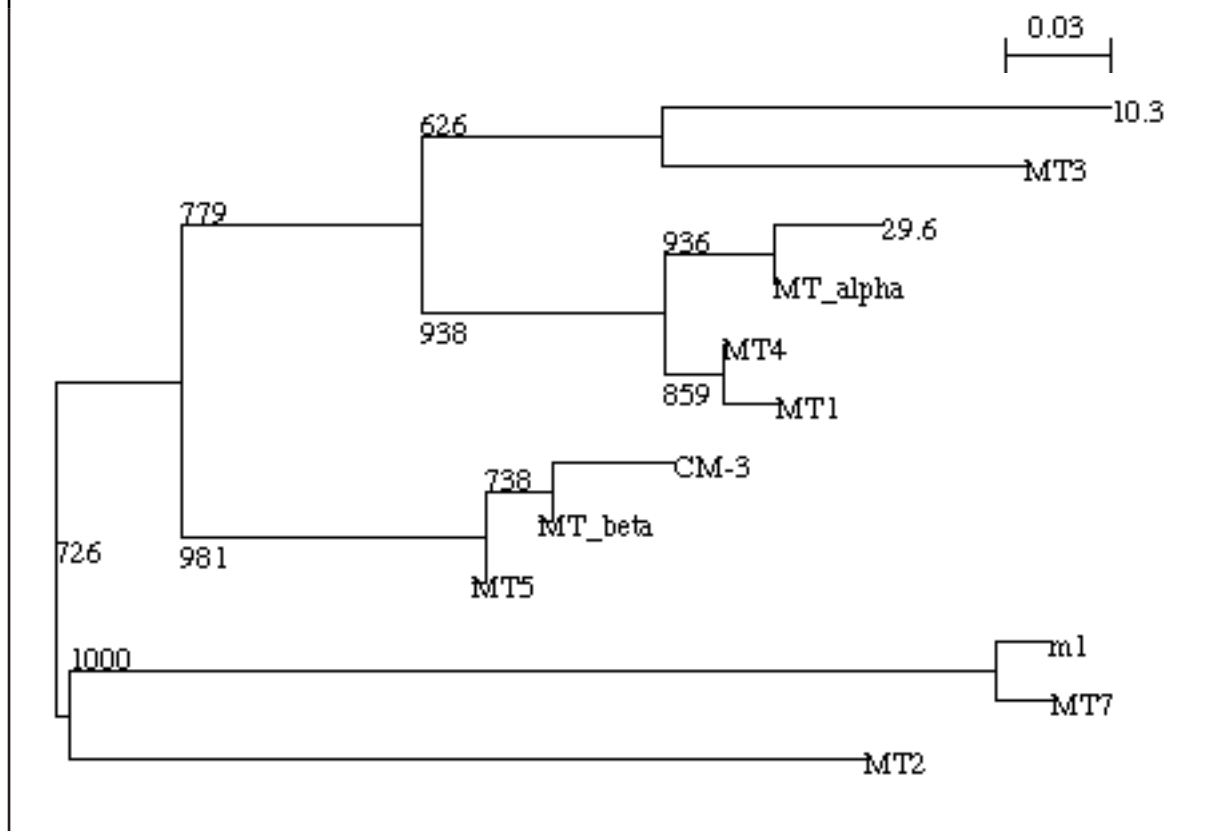
An unrooted phylogenetic tree was created with the CLUSTAL W (1.5) sequence alignment program (Thompson *et al.*, 1994), using the alignment data described in Fig. 1. Settings for the construction of the tree were:

Exclude positions with gaps: OFF Correct for multiple substitutions: ON
Bootstrap trials: 1000

A graphical representation was created with a beta version of the program NJPLOT, written by Manolo Gouy at the University of Lyon. The outgroup was selected more or less arbitrarily to facilitate identification of isotoxin groups.

CLUSTAL W (1.5) and NJPLOT are available by anonymous ftp from the EMBL server at ftp.embl-heidelberg.de/pub/software.

29.6 = DPD 29.6, 10.3 = DP II 10.3 and m1 = m1-toxin (see legend to Fig. 1).



MT 5 and MT β differ only in amino acid 48, which is Lys in MT5 (Jolkkonen, M., unpublished results; Fig. 2 of the present work) and Ile in MT β (Paper VI). MT β has moderate affinity to m3, m4 and m5 receptors (120 – 350 nM), and very low affinity to m1 and m2 (Paper VI). MT 5 binds to m1 and m4 receptors with moderate affinity, and to m3, m4 and m5 with very low affinity (Jolkkonen, M., unpublished results; Table 4 of the present work). Again, a small substitution changes the selectivity of the toxins, but this time in the third loop, which obviously also takes part in toxin binding.

MT β is also very similar to a *D. polylepis* peptide called CM-3. CM-3 has not earlier been recognised as a muscarinic toxin, and to our knowledge, the only test of its activity has been a simple assay for lethality, which proved negative even at high concentrations (Joubert, 1985).

CM-3 differs from MT β only in two positions (Fig. 2 of the present work), and it is therefore unlikely that it would not also be a muscarinic toxin.

We have not isolated any CM-3 at our laboratory – as far as we know. From the sequence of CM-3 it becomes clear that in amino acid analysis, it would be impossible to distinguish CM-3 from MT β . The molecular masses of CM-3 and MT β differ only by one unit, making it difficult to distinguish between them by mass spectrometry. It seems that the only way to distinguish between these two toxins would be by sequencing every batch of MT β produced, or possibly by a well-calibrated electrophoretic assay.

The affinities of MT1 and MT4 were found to be independent of the Arg-His substitution in position 57. Both toxins bind to m1 and m4 receptors with similar affinities, and to other subtypes with very low affinity (Jolkkonen, M., unpublished results; Table 4 of the present work).

The results obtained with MT1, showing that it has its highest affinity to both m1 and m4 receptors (Jolkkonen, M., unpublished results; Table 4 of the present work) seemed to contradict our results published in Paper I. This discrepancy is discussed in connection with Paper IX (see below).

In the experiments with cloned receptors, MT2 showed remarkably low affinity to all receptors (Table 4), as compared to the K_i value of approximately 100–200 nM obtained with synaptosomal receptors (Fig. 4 in Adem *et al.*, 1988). The latter value has low precision, due to the use of [³H]-QNB as a reporter ligand (for a discussion of experimental difficulties with QNB, see Järvi and Eller, 1988).

The difference may be due to the earlier mentioned instability of MT2. It may also have been caused by a difference in the experimental procedures, namely lack of preincubation with cloned receptors (see the discussion of papers VII and VIII). Interestingly, the affinity determined for MT2 by Jerusalinsky *et al.* (1992) with synaptosomal receptors (K_i of 23 nM to the high-affinity sites for pirenzepine, *i. e.*, m1 and m4 receptors) is also significantly higher than that determined with cloned human receptors (K_i = 364 nM for m1, 1.2 μ M for m2, and higher for m2 and m3 receptors) by Kornisiuk *et al.* (1995a; note the correction in 1995b).

Another interesting finding was that the one toxin tested, MT3, had moderate affinity (pK_i between 6 and 7) also to α_1 - and α_2 - but not to β -adrenergic receptors. This may not be very surprising, considering the homology between muscarinic and adrenergic receptors. It has, however, inspired some speculations on my part regarding such muscarinic toxins as MT2, MT5 and MT β , which in our assays show very low affinity to muscarinic receptors, be it for reasons of inactivation, as discussed above, or extremely slow binding, as discussed below. These toxins have an affinity to muscarinic receptors similar to that of MT3 to α -adrenergic receptors. They might prove to have higher affinity to adrenergic than to muscarinic receptors, and we will then have to decide on whether we should instead call them adrenergic toxins.

Table 4. Affinities of muscarinic toxins to cloned human muscarinic acetylcholine receptors expressed in Chinese hamster ovary cells. Values represent the mean \pm SD of n independent experiments. MT2 appears to be an unstable molecule. This probably explains the low affinity of the toxin preparation assayed.

K_i values for MT3 are from Paper V, for MT α and MT β from Paper VI. K_i values for m1-toxin were estimated from Fig. 11 of Max *et al.* (1993a). The K_i values for MT2, MT4, MT5, MT6 and MT7 were determined as described in Paper V (Jolkkonen, M. unpublished results).

Toxin	Receptor subtype; affinity (K_i) in nM				
	CHO-m1	CHO-m2	CHO-m3	CHO-m4	CHO-m5
<i>D. angusticeps</i>					
MT1 (n=7)	49	> 1000	> 1000	58	> 1000
MT2 (n=3)	1500	> 2000	> 2000	760	> 2000
MT3 (n=4)	78	> 1000	> 1000	2	> 1000
MT4 (n=3)	62	> 1000	> 1000	87	> 1000
MT5 (n=3)	180	> 1000	> 1000	540	> 1000
MT6 (n=1)	190	> 425	> 425	3.6	> 425
m1-toxin	0.1	no binding	no binding	9	no binding
MT7 (n=3)	0.2	> 2000	> 2000	> 2000	> 2000
<i>D. polylepis</i>					
MT α	23	44	3	5	8
MT β	> 1000	> 2000	140	120	350

The kinetics of toxin binding: Papers VII and VIII.

On the basis of some observations made while testing different methods of screening chromatographic fractions for muscarinic activity, I started to suspect that some toxins bound much more slowly than others. Further experimentation showed that, for some toxins, the extent of radioligand binding depended on the order of and interval between addition of toxin and radioligand. In order to more accurately compare the affinities of the, hypothetically, slow- and fast-acting muscarinic toxins, I therefore consequently added the toxin 60 min before the radioligand when measuring the affinity to porcine and murine (rat) receptors. This pre-incubation with toxin is described in Paper II.

Later, a more systematic study of the binding kinetics of MT2 confirmed that this toxin bound remarkably slowly to the receptor. When added simultaneously with [3 H]-NMS, MT2 in concentrations up to 7 mM had no inhibitory effect of the radioligand binding during the first ten minutes, during which time the [3 H]-NMS binding went to completion. The rate constants

of [³H]-NMS binding were determined for several toxin concentrations and were constant (Paper VII, Table 1). After longer incubation, the radioligand binding slowly started to decrease relative to a control without toxin (Paper VII, Fig. 1). It becomes clear from this figure that with simultaneous addition of MT2 and [³H]-NMS, even high concentrations of MT2 would show insignificant effects after an one hour incubation, and consequently pre-incubation with toxin is necessary when screening fractions for muscarinic activity. Since no such pre-incubation was performed when measuring the binding to cloned receptors (Paper V), this may be an alternative to the hypothesis of partial inactivation in explaining why MT2 and some other toxins showed remarkably low affinity for all subtypes of receptors (Table 4 of the present work).

We then studied the effect of pre-incubation with MT2, and found that in absence of radioligand, MT2 blocks the receptors more rapidly. Using an MT2 concentration of 7 mM, the effect was complete after one hour, and the toxin then blocked about 40 % of the receptors (Paper VII, Fig. 2). The rate of NMS binding to the receptors not blocked by MT2 remained the same as to receptors incubated without toxin. This showed that MT2 completely blocked one population of receptors while leaving the rest completely unaffected (Paper VII). This is in accordance with the selective binding of MT2 to m1 and m4 receptors (Table 4 of the present work; Kornisiuk *et al.*, 1995a; Kornisiuk *et al.*, 1995b).

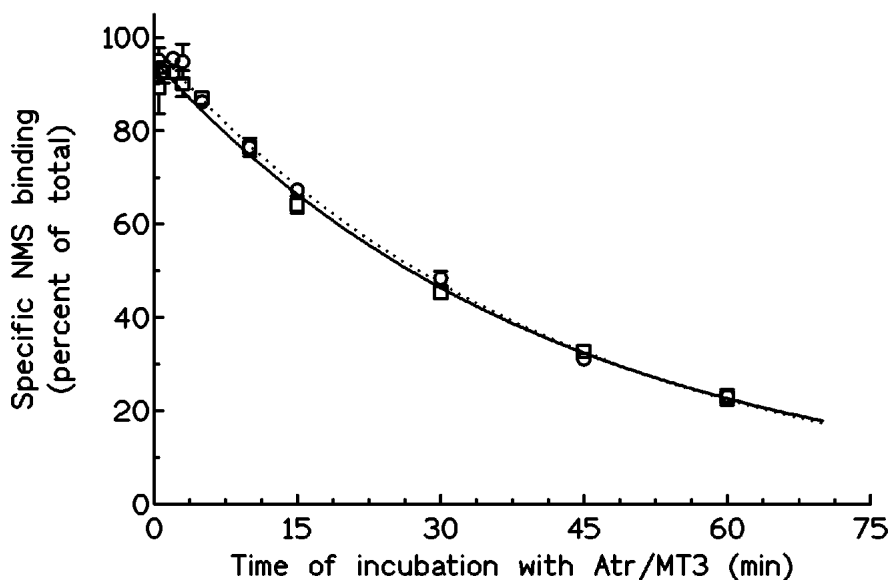
The rate constants of receptor inactivation of MT2, corresponding to the binding of MT2 in the absence of radioligand, were determined for several toxin concentrations and were found to have a hyperbolic relationship to the concentration. As shown by Strickland *et al.* (1975), such a relationship indicates that the binding of the ligand, in this case MT2, proceeds in two or several steps, where the first step is the formation of a readily reversible ligand-receptor complex, and the later steps include a slow transition of this complex to a considerably more stable form. The formation of this stable (isomerised) form had a half-life of 18 min, while the formation of the first complex was about a million times faster (Paper VI). The K_d for this first complex, as calculated from kinetic data, was 1.4 mM (not to be confused with the K_d for the isomerised complex). This means that if the toxin and NMS competed for the same binding site, the binding of MT2 would have been fast enough and the concentration of MT2 (7 mM) would have been high enough to produce significant inhibition of the NMS binding. The conclusion is that MT2 and NMS bind to different sites on the receptor, and that the isomerisation of the toxin-receptor complex, rather than the initial binding of the toxin, is incompatible with the binding of NMS to the receptor (Paper VII).

The possibility of allosteric interactions between toxin and reporter ligand further complicates the picture. It is known that muscarinic toxins may completely block the formation and dissociation of the antagonist-receptor complex (Max *et al.*, 1993b). Such strong allosteric interaction is not observed with all muscarinic toxins; for instance, 10 nM of MT3 did not influence the dissociation of [³H]-NMS from m4 receptors (Fig. 4). MT3 has its highest affinity to m4 receptors ($K_i = 2$ nM) (Table 4), and at least in this case there is no allosteric

effect on the binding of antagonist. Similar experiments with other muscarinic toxins have not yet been done, and it can not be ruled out that allosteric effects in some cases may influence the results from binding experiments.

Fig. 4. Allosteric effects of MT3. Time-course of atropine-induced dissociation of [³H]-NMS from CHO-m4 receptors in absence (○) and presence (□) of 10 nM MT3. No allosteric effect of the toxin was observed.

Method: All samples were incubated with 740 pM [³H]-NMS for one hour at room temperature. Then, after different times, atropine to a concentration of 2 μM (control samples) or atropine to a concentration of 2 μM and MT3 to a concentration of 10 nM (toxin samples) was added. The times were chosen so as to give 60, 45, 30, 15, 10, 5, 3, 2, 1 and 0.5 minutes of incubation with atropine before filtration, which was performed simultaneously with all samples. Total binding (neither atropine nor toxin added) and non-specific binding (atropine present from the beginning) were determined separately and the binding data was normalised after these values. All samples were made in duplicate and the range is shown with error bars in the graph. Preparation of receptors and determination of bound radioactivity was performed essentially as described in section 2.3 of Paper V.



For further kinetic studies of toxin binding, we chose a more fast-acting toxin, MT α . In contrast to MT2, MT α inhibited the binding of simultaneously added [³H]-NMS (Paper VIII). The rate constant for NMS binding and the amount of NMS bound at equilibrium was determined for several toxin concentrations. The curve of NMS binding versus toxin concentration gave a K_d of 210 nM for MT α (Paper VIII), which is 4 to 70 times higher than the values listed in Paper VI. The observed rate of NMS binding increased with higher toxin concentration. This is the effect of the consumption of binding sites by two parallel processes (toxin binding and NMS binding) instead of one (in the control, free binding sites are consumed only by NMS binding). The process is analogous to what would be observed if a high concentration of unlabelled NMS would be added simultaneously with [³H]-NMS; the binding goes to completion faster, and consequently the binding rate appears to be higher.

The increase in the observed binding rate of NMS in Paper VIII is nothing less than what was called the rate of inactivation of receptors by toxin in Paper VII: they are identical, although they for practical reasons were measured in different ways.

The observed rate constants for NMS binding showed a hyperbolic relation to toxin concentration, and thus MT α also binds to the receptor in a similar two-step (or possibly multi-step) fashion as MT2. The isomerisation of the MT α - receptor complex is over a hundred times faster than is the case with MT2, and is fast enough to take place on the same time-scale as the NMS binding. It is therefore not possible to draw any conclusions about the type of inhibition from the present data (Paper VIII).

Autoradiographic mapping of receptors with radiolabelled toxin MT1: Paper IX.

The results in Paper I, showing that [¹²⁵I]-MT1 bound with high affinity only to m1 receptors, encouraged us to test its potential as a tool in receptor research. The radiolabelled toxin was tested on cloned m1 and m2 receptors expressed in CHO cells and it was confirmed that it had more than 1000 times higher affinity to m1 than to m2 receptors. It was used to selectively label m1 receptors in slices of rat brain and proved its usefulness in receptor mapping. Non-specific binding, which often is a problem with radiolabelled proteins, was undetectable. The binding was compared with that of 5 nM pirenzepine. At this concentration, about 30 % of the m1 receptors, 9 % of m4 and less of other subtypes will be labelled with pirenzepine (as calculated from the dissociation constants for pirenzepine determined in Paper V: 11 nM for m1, 50 nM for m4 and higher for other subtypes). The distribution of [¹²⁵I]-MT1 binding was similar although not identical to that of pirenzepine. It was assumed that the differences were due to binding of pirenzepine to m4 receptors, while the radiolabelled toxin bound only to m1 receptors. This assumption was supported by comparing the distribution of [¹²⁵I]-MT1 binding to that of [¹²⁵I]-MT3, which selectively labels m4 receptors. The results presented in this paper confirm our original finding that [¹²⁵I]-MT1 does indeed bind only to m1 receptors, despite the fact that the native toxin has almost as high affinity to m4 as to m1 receptors.

Similar results were obtained with [¹²⁵I]-MT2, which also bound only to regions that could be labelled with low concentrations (2.5 nM) of pirenzepine (Jerusalinsky and Harvey, 1994). Note that the affinity data for m3 and m4 receptors are interchanged in the above mentioned paper, as result of an erroneously labelled shipment of receptors (Kornisiuk *et al.*, 1995b).

Obviously the iodination further restricts the specificity of the toxins. It is the tyrosine residues of a protein that incorporate the iodine, and with the moderate amounts of ¹²⁵I and short (30–60 s) reaction times used, the average incorporation is far less than one iodine atom per toxin molecule. A toxin molecule in the preparation will most likely have none of its tyrosines modified, in which case its binding is unobservable, or one iodinated tyrosine, while multiple labelling will be very rare. Consequently we will observe only the binding of single

labelled toxin.

MT1 has three Tyr residues, and it is highly unlikely that they all have the same effect of restricting the toxin's specificity when iodinated. The results indicate that one of the Tyr residues is much more susceptible to iodination than the other two, and that iodination of this residue renders the toxin unable to bind to m4 receptors. The same should be the case with MT2. The two toxins have one Tyr residue in common, Tyr30. This residue is in contact with the solvent (Ségalas, 1995a), and should therefore be easily iodinated. Tyr30 is at the tip of the second loop, which is assumed to penetrate into the ligand-binding cleft of the receptor. It is also situated next to the region 31–33 that determines selectivity in the toxin MT4, which is nearly identical to MT1. All these indications make it fairly safe to assume that it is iodination of Tyr30 which renders the toxins unable to bind to m4 receptors.

CONCLUSIONS

We have in the venom of snakes of the mamba family found ten closely related neurotoxins which bind to muscarinic acetylcholine receptors. The mamba muscarinic toxins have no known counterpart in the venoms of other snakes.

The muscarinic toxins are unique in several ways: They are the only known animal toxins specific for muscarinic receptors, the only known muscarinic ligands of peptide nature, and the only known muscarinic ligands with a high degree of subtype specificity. It may be this last property that attracts the greatest interest. It is probably the size of the toxins that enables the precise recognition of different receptor subtypes. In contrast to the low molecular weight ligands, which all have rather poor selectivity, the muscarinic toxins can interact with both the highly conserved agonist binding transmembrane regions and with the more variable extracellular domains.

We have succeeded in identifying a region of the muscarinic toxins which dramatically influences the subtype selectivity, and have also accidentally introduced a chemical modification (most likely adjacent to the above mentioned region) with similar effect. The method of site-directed mutation should be applied to pinpoint down the effects of single substitutions in this area. I hope to find opportunity to perform these experiments.

Although we have performed rather few physiological experiments, our results support the existing reports of muscarinic toxins (with the exception of m1-toxin) being agonistic in their actions.

The binding of muscarinic toxins has been shown to take place in two discreet steps, the first being the physical association of free toxin to the receptor, and the other being an internal reorganisation of the toxin-receptor complex into a more stable form. Both the toxins used in these studies have agonist-like effects, and the second step may be equivalent to the conformational change in the receptor that normally follows agonist binding and transduces the signal across the membrane. The slowness of this step (from over ten minutes to about ten seconds depending on which toxin is used) may be due to the presumably large number of intermolecular bonds that have to be rearranged, as compared to the case with smaller ligands like acetylcholine.

The kinetic results (Paper VII) indicate that MT2 and NMS bind to different sites and may form a ternary toxin-antagonist-receptor complex. It may be difficult to envision how an antagonist could bind or remain bound to the receptor while it is occupied by such a huge ligand as a muscarinic toxin, especially if the second loop of MT2 and other muscarinic toxins indeed penetrates down to the agonist-binding site as hypothesised in Paper VI. We do, however, know that in the case of m1-toxin, a toxin-NMS-receptor complex can form (Max *et al.*, 1993b). The two cases are probably not completely equivalent; for instance, m1-toxin is reported to be an antagonist, while MT2 seems to be an agonist (Jerusalinsky and Harvey, 1994).

The many questions surrounding the allosteric effects and ternary complexes involving toxin and low molecular ligands probably require some experimental data on the structure of the muscarinic toxin-receptor complex before they can be solved. Such data would also be helpful in explaining the subtype selectivity of the toxins and understanding receptor function, and might be of value in the design of synthetic ligands and pharmaceuticals.

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Biochemistry, University of Uppsala, Uppsala, Sweden. I wish to express my thanks to the following persons:

At the Biomedical Center, Uppsala University,

Dr. Evert Karlsson, supervisor and friend.

Dr. Carlos Červeňanský, for good companionship and for innumerable references and interesting unpublished observations.

Dr. Christer Wernstedt and Dr. Ulf Hellman, for spending their valuable time on our peculiar and problematic peptide fragments.

Dr. Åke Engström for help with mass spectrometry.

Dr. Ulf Ragnarsson, for many constructive suggestions and for help in establishing cooperation with Tartu University.

Dr. David Eaker, for the linguistic revision of this thesis.

Uno Skatt and Hannu Ukkonen, for help with the production of graphics and for trusting me with their computers.

Bengt Westerlund, for practical help with the final stages of preparing the manuscript.

Lilian Forsberg, for the use of equipment from the student's laboratory.

All others of the staff who have taken an interest in my research and thus encouraged me to carry on my studies despite the lack of official economic support towards the end.

At the Marion Merrell Dow Research Institute, Strasbourg,

Dr. Paul van Gierbergen, Dr. Luc Van Hijfte and Dr. Mary Richardson, without whose help and interest in my work this thesis had been much shorter.

Dr. Pierre Lepage, for valuable aid with mass spectrometry.

Matthieu Poirot and Cathy Chedal, without whom my impression of Strasbourg would have been rather different.

At the Institute of Chemical Physics, Tartu University,

Prof. Jaak Järv, for teaching me new methods of research and improving my understanding of scientific thinking and the design of experiments.

Martin Lepiku, Aldo Oras, Ursel Soomets, Tanel Toomela and others, for their assistance and contributions in and outside of the laboratory.

At BioCity, Åbo Academy,

Dr. Rasmus Hautala and Prof. Kalle Åkerman, for their help with pharmacological investigations that unfortunately have not yet resulted in a paper.

And at S:t Eskils Högre Allmänna Läroverk, Eskilstuna,

Sven Lindström, for taking his profession as a teacher seriously, and doing an excellent job at it.

I also wish to thank all the co-authors of the papers in this thesis for the good co-operation and hope that we will continue our mutual investigations in the future.

The amino acid sequences of *D. polylepis* peptides DP II 10.3 and DPD 29.6 are included by courtesy of Dr. Leonard A. Smith, Toxinology Division of the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, U.S.A.

My thanks to those outside the field of chemistry will be conveyed in person.

ERRATA

Paper I, page 152: "The m1 subclass corresponds probably to M1, m2 to M2 and m3 to M3. Subtype M1 dominates in brain, M2 in smooth muscle and M3 in cardiac muscle (Levine and Birdsall, 1989)."

The text should read "Subtype M1 dominates in brain, M2 in cardiac muscle and M3 in smooth muscle".

Paper II, page 404: "Cortex has a high receptor density and m1 receptors account for 35 %, m2 and m3 for 11 % each, and m4 for 43 % (Waelbroeck *et al.*, 1990)."

Table 3 from Waelbroeck *et al.* (1990) was misinterpreted. Although the ratios quoted are wrong, this lacks importance for the conclusions drawn. See Table 2 of the present work for more data on receptor ratios in cortex, including the correct values from Waelbroeck *et al.*, 1990.

Paper I, pages 521 and 524. The formula weight of MT2 is reported to be 7040. The correctly calculated mass is 7076.1.

Paper VII, page 97: "The half-life for isomerization of RT to (RT) is 18 min as calculated from the rate constant k_2 ." The correct value of the half-life is 14 min.

APPENDIX

Molecular masses and molar absorptions of muscarinic toxins.

Molecular masses (Mr) are calculated from the composition assuming four disulphide bonds. When the sequence is unknown, the mass is calculated assuming no amidation of acidic residues. Experimentally determined molar absorptions (A^M) are given at absorption maximum.

D. angusticeps toxins

	MT1	MT2	MT3	MT4	MT5	MT6	MT7
Residues	66	65	65	66	65	65	65
Sequence known	yes	yes	yes	yes	yes	no	no
Mr, composition	7509.7	7076.1	7379.5	7490	7356	7328	7472
Mr, mass spectrometry	7509.4	7077.0	7379.7	7490.1	7352.0	7323.0	no data
UV abs. max.	276 nm	276 nm	276 nm	278 nm	276 nm	274 nm	276 nm
A^M , $M^{-1}cm^{-1}$	16000	10400	10600	16700	5200	10900	15900

D. polylepis toxins

	MT α	MT β	MT γ
Residues	66	65	65
Sequence known	yes	yes	no
Mr, composition	7545.0	7337.5	7437.0
Mr, mass spectrometry	7546.0	7336.7	no data
UV abs. max.	280 nm	276 nm	278 nm
A^M , $M^{-1}cm^{-1}$	15900	5600	18200

REFERENCES

- Adem, A., Åsblom, A., Johansson, G., Mbugua, P. M. and Karlsson, E. (1988) Toxins from the venom of the green mamba *Dendroaspis angusticeps* that inhibit the binding of quinuclidinyl benzilate to muscarinic acetylcholine receptors. *Biochim. biophys. Acta* **968**, 340–345.
- Akiba, I., Kubo, T., Maeda, A., Bujo, H., Nakai, J., Mishina, M. and Numa, S. (1988) Primary structure of porcine muscarinic acetylcholine receptor III and antagonist binding studies. *FEBS Lett.* **235**, 257–261.
- Albrand, J.-P., Blackledge, M. J., Pascaud, F., Hollecker, M. and Marion, D. (1995) NMR and restrained molecular dynamics study of the three-dimensional solution structure of toxin FS2, a specific blocker of the L-type calcium channel, isolated from black mamba venom. *Biochemistry* **34**, 5923–5937.
- Anwar-ul, S., Gilani, H. and Cobbin, L. B. (1986) The cardio-selectivity of himbacine: a muscarine receptor antagonist. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**, 16–20.
- Brann, M. R., Buckley, N. J., Jones, S. V. and Bonner, T. I. (1987) Expression of a cloned muscarinic receptor in A9 L cells. *Mol. Pharmacol.* **32**, 450–455.
- Birnbaumer *et al.* (1987) quoted in Nicholls, J. G., Martin, A. R. and Wallace, B. G. (1992) *From Neuron to Brain*. Sinauer Associates, Inc. Sunderland, Massachusetts.
- Bonner, T. I. (1989) The molecular basis of muscarinic receptor diversity. *Trends Neurosci.* **12**, 148–151.
- Bowman, W. C. (1990) *Pharmacology of neuromuscular function, 2nd ed.* Wright, London.
- Caulfield, M.P. (1993) Muscarinic receptors – characterization, coupling and function. *Pharmac. Ther.* **58**, 319–379.
- Červeňanský, C., Dajas, F., Harvey, A.L. and Karlsson, E. (1991) Fasciculins, anticholinesterase toxins from mamba venoms: Biochemistry and pharmacology, in *International Encyclopedia of Pharmacology and Therapeutics, Snake Toxins* (Harvey, A.L., ed.) pp. 303–321. Pergamon Press, New York.
- Chapman, D. S. (1968) The symptomatology, pathology and treatment of the bites of venomous snakes of Central and South Africa, in *Venomous Animals and Their Venoms* (Bücherl, W., Buckley, E. E. and Deulofeu, V., eds.) pp. 463–527. Academic, New York.
- Cheng, Y. C. and Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 % inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108.
- Chiappinelli, V. A. (1991) κ -neurotoxins and α -neurotoxins: Effects on neuronal nicotinic acetylcholine receptors, in *International Encyclopedia of Pharmacology and Therapeutics, Snake Toxins* (Harvey, A.L., ed.) pp. 223–258. Pergamon Press, New York.
- Dale, H. H. (1914) The action of certain esters and ethers of choline, and their relation to muscarine. *J. Pharmacol. Exp. Ther.* **6**, 147–190.

- Dev, N. B. and Loeschcke, H. H. (1979) A cholinergic mechanism involved in the respiratory chemosensitivity of medulla oblongata in the cat. *Pflügers Arch.* **379**, 29–36.
- De Weille, J. R., Schweitz, H., Maes, P., Tartar, A. and Lazdunski, M. (1991) Calciseptine, a peptide isolated from black mamba venom, is a potent specific blocker of the L-type calcium channel. *Proc. Natl. Acad. Sci. USA* **88**, 2437–2440.
- DiPaola, M., Czajkowski, C. and Karlin, A. (1989) The sidedness of the COOH terminus of the acetylcholine receptor δ subunit. *J. Biol. Chem.* **264**, 15457–15463.
- Durán, R., Červeňanský, C., Dajas, F. and Tipton, K. (1994) Fasciculin inhibition of acetylcholinesterase is prevented by chemical modification of the enzyme at a peripheral site. *Biochim. Biophys. Acta* **1201**, 381–388.
- Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. and Brann, M.R. (1991) Antagonist binding profiles of five cloned muscarinic receptor subtypes. *J. Pharm. Exp. Ther.* **256**, 727–733.
- Giuliano, R., Ruggiero, D. A., Morrison, S., Ernsberger, P. and Reis, D. J. (1989) Cholinergic regulation of arterial pressure by the C1 area of the rostral ventrolateral medulla. *J. Neurosci.* **9**, 923–942.
- Gocayne, J., Robinson, D. A., FitzGerald, M. G., Chung, F. Z., Kerlavage, A. R., Lentes, K. U., Lai, J., Wang, C. D., Fraser, C. M. and Venter, J. C. (1987) Primary structure of rat cardiac beta-adrenergic and muscarinic cholinergic receptors obtained by automated DNA sequence analysis: further evidence for a multigene family. *Proc Natl Acad Sci USA* **84**, 8296–8300.
- Goyal, R. K. (1989) Muscarinic receptor subtypes. Physiology and clinical implications. *New England J. Medicine* **321**, 1022–1029.
- Harvey, A. L. and Tamiya, N. (1980) Role of phospholipase A activity in the neuromuscular paralysis produced by some components isolated from the venom of the sea snake, *Laticauda semifasciata*. *Toxicon* **18**, 65–69.
- Harvey, A.L., Andersson, A.J., Mgubua, P. M. and Karlsson, E (1984) Toxins from mamba venoms that facilitate neuromuscular transmission. *J. Toxicol. Toxin Rev.* **3**, 91–137.
- Harvey, A.L. and Anderson, A.J. (1991) Dendrotoxins: Snake toxins that block potassium channels and facilitate transmitter release, in *International Encyclopedia of Pharmacology and Therapeutics, Snake Toxins* (Harvey, A.L., ed.) pp. 131–164. Pergamon Press, New York.
- Izquierdo, I. (1989) Mechanism of action of scopolamine as an amnesic. *Trends Pharmacol. Sci.* **10**, 175–177.
- Jerusalinsky, D., Červeňanský, C., Peña, C., Raskovsky, S. and Dajas, F. (1992) Two polypeptides from *Dendroaspis angusticeps* venom selectively inhibit the binding of central muscarinic cholinergic receptor ligands. *Neurochem. Int.* **20**, 237–246.

- Jerusalinsky, D., Červeňanský, C., Walz, R., Bianchin, M. and Izquierdo, I. (1993) A peptide muscarinic toxin from the green mamba venom shows agonist-like action in an inhibitory avoidance learning task. *Eur. J. Pharmacol.* **240**, 103–105.
- Jerusalinsky, D. and Harvey, A. L. (1994) Toxins from mamba venoms: small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **15**, 424–430.
- Joubert, F. J. (1985) The amino acid sequence of protein CM-3 from *Dendroaspis polylepis polylepis* (black mamba). *Int. J. Biochem.* **17**, 695–699.
- Järv, J. and Eller, M. (1988) Kinetic aspects of L-quinuclidinyl benzilate interaction with muscarinic receptor. *Neurochem. Int.* **13**, 419–428.
- Kauffeld, C. F. (1963) More at home with cobras. *Philadelphia Herpetological Society Bulletin* **11**, 13–18.
- Kornisiuk, E., Jerusalinsky, D., Červeňanský, C. and Harvey, A. L. (1995a) Binding of muscarinic toxins MTx1 and MTx2 from the venom of the green mamba *Dendroaspis angusticeps* to cloned human muscarinic cholinergic receptors. *Toxicon* **33**, 11–18.
- Kornisiuk, E., Jerusalinsky, D., Červeňanský, C. and Harvey, A. L. (1995b) Corrigendum. *Toxicon* **33**, 1111.
- Levey, A. I., Kitt, C. A., Simonds, W. F., Price, D. L. and Brann, M. R. (1991) Identification and localisation of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci.* **11**, 3218–3226.
- Li, M., Yasuda, R. P., Wall, S. J., Wellstein, A. and Wolfe, B. B. (1991) Distribution of m2 muscarinic receptors in rat brain using antisera selective for m2 receptors. *Mol. Pharmacol.* **40**, 28–35.
- Marchot, P., Khélif, A., Ji, Y.H., Mansuelle, P. and Bougis, P. (1993) Binding of ¹²⁵I-fasciculin to rat brain acetylcholinesterase. *J. Biol. Chem.* **268**, 12456–12467.
- Max, S. I., Liang, J. S. and Potter, L. T. (1993a) Purification and properties of m1-toxin, a specific antagonist of m1 muscarinic receptors. *J. Neurosci.* **13**, 4293–4300.
- Max, S. I., Liang, J. S. and Potter, L. T. (1993b) Stable allosteric binding of m1-toxin to m1 muscarinic receptors. *Mol. Pharmacol.* **44**, 1171–1175.
- Mayanil, C. S., Richardson, R. M. and Hosey, M. M. (1991) Subtype-specific antibodies for muscarinic cholinergic receptors. I. Characterization using transfected cells and avian and mammalian cardiac membranes. *Mol. Pharmacol.* **40**, 900–907.
- McCrea, P. D., Popot, J.-L. and Engelman, D. M. (1987) Transmembrane topography of the nicotinic acetylcholine receptor delta subunit. *EMBO J.* **6**, 3619–3626.
- McGehee, D. S., Heath, M. J. S., Gelber, S., Devay, P. and Role, L. W. (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science* **269**, 1692–1696.

- Mebs, D. and Claus, I. (1991) Amino acid sequences and toxicities of snake venom components, in *International Encyclopedia of Pharmacology and Therapeutics, Snake Toxins* (Harvey, A. L., ed.) pp. 425–447, Pergamon Press, New York.
- Meselson, M and Robinson, J. P. (1980) Chemical warfare and chemical disarmament. *Sci. Am.* **242**, pp 34–43.
- Metzner, R. (1970) Mushrooms and the mind, in *Psychedelics: The Uses and Implications of Hallucinogenic Drugs* (Aaronson, B. and Osmond, H., eds.) pp. 90–107, Doubleday & Company, Inc., New York.
- Mullins, L. J. (1975) Ion selectivity of carriers and channels. *Biophys. J.* **15**, 921–931.
- Nattie, E. E., Wood, J., Mega, A. and Goritski, W. (1989) Rostral ventrolateral medulla muscarinic receptor involvement in central ventilatory chemosensitivity. *J Appl. Physiol.* **66**, 1462–1470.
- Peralta, E. G., Ashkenazi, A., Winslow, J. W., Smith, D. H., Ramachandran, J. and Capon, D. J. (1987) Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.* **6**, 3923–3929.
- Perkins, J. R., Parker, C. E. and Tomer, K. B. (1993) The characterization of snake venoms using capillary electrophoresis in conjunction with electrospray mass spectrometry: Black mambas. *Electrophoresis* **14**, 458–468.
- Picciotto, M. R., Zoli, M., Léna, C., Bessis, A., Lallemand, Y., LeNovère, N., Vincent, P., Pich, E. M., Brûlet, P. and Changeux, J.-P. (1995) Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. *Nature* **374**, 65–67.
- Puu, G. and Koch, M. (1990) Comparison of kinetic parameters for acetylthiocholine, soman, ketamine and fasciculin towards acetylcholinesterase in liposomes and in solution. *Biochem. Pharmacol.* **40**, 2209–2214.
- Radic, Z., Durán, R., Vellom, D.C., Li, Y., Červeňanský, C. and Taylor, P. (1994) Site of fasciculin interaction with acetylcholinesterase. *J. Biol. Chem.* **269**, 11233–11239.
- Rodríguez-Ithurrealde, D., Silveira, R., Barbeito, L. and Dajas, F. (1983) Fasciculin, powerful anticholinesterase polypeptide from *Dendroaspis angusticeps* venom. *Neurochem. Int.* **5**, 267–274.
- Saitou N., and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425.
- Sakmann, B., Noma, A. and Trautwein, W. (1983) Acetylcholine activation of single muscarinic K⁺ channels in isolated pacemaker cells of the mammalian heart. *Nature* **303**, 250–253.
- Sargent, P. B. (1993) The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* **16**, 403–443.

- Schweitz, H., Heurteaux, C., Bois, P., Moinier, D., Romey, G. and Lazdunski, M. (1994) Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, is a potent blocker of high threshold Ca²⁺ channels with a high affinity for L-type channels in cerebellar granule neurons. *Proc. Natl. Acad. Sci. USA* **91**, 878–882.
- Ségalas, I., Roumestand, C., Zinn-Justin, S., Gilquin, B., Ménez, R., Ménez, A. and Toma, F. (1995a) Solution structure of a green mamba toxin that activates muscarinic acetylcholine receptors, as studied by nuclear magnetic resonance and molecular modeling. *Biochemistry* **34**, 1248–1260.
- Ségalas, I., Thai, R., Ménez, R. and Vita, C. (1995b) A particularly labile Asp–Pro bond in the green mamba muscarinic toxin MTX2. Effect of protein conformation on the rate of cleavage. *FEBS Lett.* **371**, 171–175.
- Shulgin, A. T. (1982) Chemistry of psychotomimetics, in *Handbook of Experimental Pharmacology*, vol. 55/III (Hoffmeister, F. and Stille, G., eds.) pp. 17–18, Springer-Verlag, Berlin.
- Sivilotti, L. and Colquhoun, D. (1995) Acetylcholine receptors : Too many channels, too few functions. *Science* **269**, 1681–1682.
- Sollmann, T. (1957) *A manual of pharmacology and its applications to therapeutics and toxicology, eight edition*, pp 410–411. W. B. Saunders Company, Philadelphia.
- Strickland, S., Palmer, G. and Massey, V. (1975) Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data. *J. Biol. Chem.* **250**, 4048–4052.
- Sutcliffe, M. J., Jaseja, M., Hyde, E. I., Lu, X. and Williams, J. A. (1994) Three-dimensional structure of the RGD-containing neurotoxin homologue dendroaspin. *Struct. Biol.* **1**, 802–807.
- Taylor, P. (1980) Cholinergic agonists, in *The pharmacological basis of therapeutics* (Goodman Gilman, A., Goodman, L. S. and Gilman, A. eds.) pp 91–99. Macmillan Publishing Co., Inc., New York.
- Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.
- Wall, S. J., Yasuda, R. P., Hory, F., Flagg, S., Martin, B. M., Ginns, E. I. and Wolfe, B. B. (1991a) Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. *Mol. Pharmacol.* **39**, 643–649.
- Wall, S. J., Yasuda, R. P., Li, M. and Wolfe, B. B. (1991b) Development of an antiserum against m3 muscarinic receptors: distribution of m3 receptors in rat tissues and clonal cell lines. *Mol. Pharmacol.* **40**, 783–789.

- Weiner, N.(1980) Atropine, scopolamine, and related antimuscarinic drugs, in *The pharmacological basis of therapeutics* (Goodman Gilman, A., Goodman, L. S. and Gilman, A. eds.) pp 121–137. Macmillan Publishing Co., Inc., New York.
- Welsh, J. H. (1967) Acetylcholine in snake venoms, in *Animal Toxins* (Russell, F. E. and Saunders, P.R., Eds.) pp. 363–368, Pergamon, New York.
- Yasuda, R. P., Ciesla, W., Flores, L. R., Wall, S. J., Li, M., Satkus, S. A., Weisstein, J. S., Spagnola, B. V. and Wolfe, B. B. (1993) Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: Distribution of m4 and m5 receptors in rat brain. *Mol. Pharmacol.* **43**, 149–157.

CORRIGENDUM

Page	Line	Reads	Should read
32	14	Fig. 2 of the present work	Fig. 1 of the present work
13	16	Ligand	Ligands
19	18	relations	relationships
19	20	eight	eighth
19	31	results with	results obtained with
20	28	if a molecule	whether a molecule
25	29	what subtypes	which subtypes
26	8–9	contrarily to the popular	contrary to popular
26	12	like the use	such as the use
26	27	retain all of its activity	persist
26	32	overlapping	overlap
40	21	discreet	discrete

As will be obvious from the above corrections and several other linguistic shortcomings in the text, the manuscript was sent to print without the linguistic revisions kindly performed by Dr. David Eaker. I apologise for the fact that the Acknowledgements list erroneously points out Dr. Eaker as responsible for the proofreading of the manuscript.

Due to an unfortunate error in the typographic process, all Greek letters in the published (FEBS Letters) version of Paper VII have been converted to their Latin counterparts. This affects terms such as α -neurotoxin, κ -neurotoxin and, more seriously, the prefix μ (micro-), which appears as m (milli-). This has in turn led to corresponding errors in the discussion of this paper. They appear on page 35, line 12, and page 36, lines 12, 27 and 29. In all these cases, the concentration should be given in μM , not in mM.

ADDENDUM

Page 3: The full reference to Paper VI is

- VI. Jolkkonen, M., van Giersbergen, P.L.M., Hellman, U., Wernstedt, C., Oras, A., Satyapan, N., Adem, A. and Karlsson, E. (1995)
Muscarinic toxins from the black mamba *Dendroaspis polylepis*.
Eur. J. Biochem. **234**, 579–585.

As for the stating of 1994 as publication year for this paper, quoted as "in press" in 1995, I can only explain it as a curious writing error.