Ion selectivity and membrane potential effects of two scorpion pore-forming peptides

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Table of contents

Preface

It would like to convey to the reader that it was chosen to compile this dissertation in article format. Chapter 3 and 4 are manuscripts written as articles in accordance with the format required by the journal to which it was submitted. Each article includes a brief literature study containing literature relevant to the topic/s of concern as well as the results obtained. A more in depth literature study is compiled in Chapter 1. Chapter 4 provides an overall conclusion of both articles as well as recommendations for further studies.

The article titled *"Ion selectivity of scorpion toxin-induced pores in cardiac myocytes"* was submitted to the Elsevier journal Peptides for peer reviewing on 4 May 2005 and accepted for publication on 24 June 2005. The second article titled *"A confocal microscopy study of membrane potential changes induced by scorpion pore-forming toxins"* is to be submitted to the Elsevier Journal Toxicon for peer reviewing.

Authors' contributions

Authors	Contributions
Dale Elgar	• Responsible for literature searches, interpretation of data and writing of the articles. • Patch-clamping experimentation: Na^+ and K^+ substitution experiments of parabutoporin and opistoporin 1 and the effect of gramicidin A on cardiac myocytes. • Osmotic protection assay of opistoporin 1. • Confocal microscopy: effect of parabutoporin and opistoporin 1 on membrane potential changes in cardiac myocytes and neuroblastoma cell line.
Fons Verdonck	• Co-supervisor to the degree M.Sc (Physiology). • Donation of the chemically synthesized parabutoporin and opistoporin 1 used in the above-mentioned articles.
Anne Grobler	• Confocal microscopy expertise. • Assistance with the protocol used to investigate changes in membrane potential.
Carla Fourie	• Isolation of the cardiac myocytes. • Osmotic protection assay of parabutoporin.
Johan Du Plessis	• Supervisor to the degree M.Sc (Physiology). • Patch clamping experimentation: Cl^- substitution in the presence of parabutoporin.

The following is a statement from the co-authors that confirms each individual's role in the study:
I declare that the above-mentioned articles are approved and that my role in the study, stated above, is representative of my actual contributions. I hereby give consent that they may be published as part of Dale Elgar's M.Sc (Physiology) dissertation.

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(Supervisor)

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Mrs. C.M.T. Fourie

Mrs. A. Grobler

List of Abbreviations

$BeIT_5A$	- insect toxin from *Buthus eupeus*
BmKa1 and 2	- *Buthus martensii* Karsch linear α-helical antimicrobial peptides
BmKb1	- *Buthus martensii* Karsch linear α-helical antimicrobial peptide
BmKbpp	- *Buthus martensii* Karsch bradykinin potentiating peptide
BmKn1 and 2	- *Buthus martensii* Karsch linear α-helical antimicrobial peptides
BmTXKS2	- *Buthus martensii* Karsch linear α-helical antimicrobial peptide
C-terminal	- carboxyl terminal
Ca^{2+}	- calcium ion
$[Ca^{2+}]_i$	- intracellular calcium concentration
Cl^-	- chloride ion
Cs^+	- cesium
Da	- Dalton
ED_{50}	- half maximum effective dose
EGTA	- ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
E_K	- equilibrium potential of K^+
E_{rev}	- reversal potential
$Flourescence_{extra}$	- extracellular fluorophore fluorescence
$Flourescence_{intra}$	- intracellular fluorophore fluorescence
$[Fluorophore]_{extra}$	- extracellular fluorophore concentration
$[Fluorophore]_{intra}$	- intracellular fluorophore concentration
H^+	- hydrogen ion
HD_{50}	- half maximum hemolytic dose
HEPES	- 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonicacid
$[ion^+]_e$	- extracellular ion concentration
$[ion^+]_i$	- intracellular ion concentration
$IpTx_a$	- Imperatoxin A
$IpTx_i$	- Imperatoxin I
IsCT	- *Opisthacanthus madagascariensis* linear α-helical antimicrobial peptide
$IsCT_2$	- *Opisthacanthus madagascariensis* linear α-helical antimicrobial peptide
K^+	- potassium ion
kDa	- kilo Dalton

Li^+	- lithium ion
Lqdef	- defensin in the hemolymph of *Leiurus quinquestriatus*
MP	- membrane potential
µg/ml	- microgram per millilitre
µm	- micrometer
µM	- micromolar
mM	- millimolar
mV	- millivolt
MIC	- minimal inhibitory concentration
N-terminal	- amino terminal
Na^+	- sodium ion
NH_4^+	- ammonium ion
$NMDG^+$	- *N*-methyl-D-glucamine ion
OP1	- opistoporin 1
OP2	- opistoporin 2
PBS	- phosphate buffer solution
PLA_2	- phospholipase A_2
PP	- parabutoporin
Rb^+	- rubidium ion
RYR	- ryanodine receptor
TFE	- trifluorethanol
TMRE	- tetramethylrhodamine ethylester
TMRM	- tetramethylrhodamine methylester

List of Figures and Tables

Page

Chapter 2

Chapter 3

Chapter 4

Summary

Parabutoporin (PP) and opistoporin 1 (OP1) are cation, α-helical antimicrobial peptides isolated from the southern African scorpion species, *Parabuthus schlechteri* and *Opistophthalmus carinatus*, respectively. Along with their antimicrobial action against bacteria and fungi, these peptides show pore-forming properties in the membranes of mammalian cells. Pore-formation and ion selectivity in cardiac myocytes were investigated by measuring the whole cell leak current by means of the patch clamp technique. Pore-formation was observed as the induction of leak currents. Ion selectivity of the pores was indicated by the shift of the reversal potential (E_{rev}) upon substitution of intra (K^+ with Cs^+ and Cl^- with aspartate) and extracellular (Na^+ with $NMDG^+$) ions. Results were compared with the effect of gramicidin A used as a positive control for monovalent cation selective pores. PP and OP1 induced a fluctuating leak current and indicate non-selectivity of PP and OP1-induced pores. An osmotic protection assay to determine estimated pore size was performed on the cardiac myocytes. PP and OP1-induced pores had an estimate pore size of 1.38-1.78 nm in diameter.

The effect of PP and OP1 on the membrane potential (MP) of a neuroblastoma cell line and cardiac myocytes was investigated. TMRM was used to mark the MP fluorescently and a confocal microscope used to record the data digitally. The resting membrane potential (RMP) of the neuroblastoma cells was calculated at -38.3 ± 1.9 mV. PP (0.5 μM) and OP1 (0.5-1 μM) depolarized the entire cell uniformly to a MP of -11.9 ± 3.9 mV and -9.4 ± 1.9 mV, respectively. This occurred after 20-30 min of peptide exposure. In the case of the cardiac myocytes depolarization was induced to -39.7 ± 8.4 mV and -32.6 ± 5.2 mV by 0.5-1 μM PP and 1.5-2.5 μM OP1, respectively.

Keywords

Scorpion toxins, antimicrobial peptides, pore-formation, ion selectivity, pore size, membrane potential.

Opsomming

Parabutoporien (PP) en Opistoporien (OP1) is kationiese, α-heliese antimikrobiese peptiede wat onderskeidelik uit die venoom van die suider-Afrikaanse skerpioenspesies, *Parabuthus schlechteri* en *Opistophthalmus carinatus* geïsoleer is. Tesame met hul antimikrobiese werking teen bakterieë en fungi, vertoon die peptiede porie vormende eienskappe in die membrane van soogdierselle. Porievorming en ioonselektiwiteit in kardiale miosiete is ondersoek deur van die heelselspanningsklemmingtegniek gebruik te maak. Porie vorming is waargeneem as die indusering van lekstrome. Ioonselektiwiteit van porieë is aangetoon deur die verskuiwing van die omkeerpotensiaal (E_{rev}) tydens die vervanging van intra (K^+ met Cs^+ en Cl^- met aspartaat) en ekstrasellulêr (Na^+ met $NMDG^+$) ione. Resultate is vergelyk met die effek van gramisidien A, wat as positiewe kontrole vir monovalente katioonselektiewe porieë gebruik is. PP en OP1 veroorsaak 'n fluktuerende lekstroom en toon non-selektiwiteit van PP en OP1 geïnduseerde porieë aan. 'n Osmotiese beskermingstoets is uitgevoer om die geskatte porie grootte in kardiale miosiete vas te stel. PP en OP1 geïnduseerde porieë het 'n geskatte porie grootte van 1,38 – 1,78 nm.

Die effek van PP en OP1 op die membraanpotensiaal (MP) van 'n neuroblastoomsellyn en kardiale miosiete is ook ondersoek. TMRM is gebruik om die MP fluoriserend te merk en 'n konfokale mikroskoop is gebruik om die data digitaal vas te vang. Die rustende membraanpotensiaal (RMP) van neuroblastoomselle was -38,3 ± 1,9 mV. PP (0,5 μM) en OP1 (0,5-1 μM) het selle eenvormig gedepolariseer tot 'n MP van -11,9 ± 3,9 mV en -9,4 ± 1,9 mV, 20 – 30 minute na peptiedblootstelling, onderskeidelik. Met kardiale miosiete is depolarisasie onderskeidelik geïnduseer tot -39,7 ± 8,4 mV en -32,6 ± 5,2 mV deur 0,5-1 μM PP and 1,5-2,5 μM OP1.

Trefwoorde

skerpioentoksiene, antimikrobiese peptiede, porie vorming, ioonselektiwiteit, porie grootte, membraanpotensiaal.

Chapter1: General Introduction

1. Introduction

Envenomations by poisonous animals, including scorpions, have been scourges of humankind since antiquity and have prompted numerous investigations to determine the mechanisms of toxicity. At present scorpion venoms are described as being diverse mixtures of predominantly peptide toxins targeting Na^+, K^+, Ca^{2+} and Cl^- channels (Tytgat *et al.*, 1999:444; Possani *et al.*, 2000:861; Olamendi-Portugal *et al.*, 2002:562). More recently a unique group of peptide toxins has been found to interact with mammalian, bacterial and fungal membranes, namely the antimicrobial or pore-forming peptides (Verdonck *et al.*, 2000:247, Torres-Larios *et al.*, 2000:5023). These peptides form part of the innate immunity of a variety of animals and it can be said that they serve as "nature's source of antibiotics" (Hancock, 2001:156; Vizioli & Salzet, 2002:494). In the venom of different animals these peptides serve as defensive and offensive weapons, enabling the deterring of predators and the capturing of prey respectively.

This group of peptides found in scorpion venom have unique amino acid sequences and form distinct structures when incorporated in the cell membrane. Peptides have either an over-representation of certain amino acids, cysteine containing or cysteine-free structures, with the later leading to the formation of cation, linear α-helical structures in the membranes (Conde *et al.*, 2000:165; Moerman *et al.*, 2002:4799).

Two peptides from southern African scorpion species namely parabutoporin (PP), from *Parabuthus. schlechteri* Purcell and opistoporin 1 (OP1), from *Opistophtalmus carinatus* Peters, 1861, are cysteine-free peptides and have been isolated and characterized. PP and OP1 have shown to have a spectrum of effects on a variety of different cell types. Both peptides have been shown to inhibit the growth of Gram-negative and Gram-positive bacteria and fungus (Moerman *et al.*, 2000:4804). At submicromolar concentrations these peptides have the ability to release Ca^{2+} from intracellular stores by a G-protein mediated pathway (Moerman *et al.*, 2003:90) and PP has been reported to inhibit (by suppression of the NADPH oxidase via a Rac activation pathway) and activate human granulocytes (by stimulating exocytosis and chemotaxis) (Willems *et al.*, 2002:1683).

Another property associated with linear α-helical peptides is the formation of transmembrane pores allowing for the trafficking of ions in and out of the cell. PP has been shown to induce these pores in rat dorsal root ganglion cells (Verdonck *et al.*, 2000:255). Similarly, micromolar concentrations of OP1 were reported to cause a larger increase in intracellular Ca^{2+} concentration in the presence rather than in the absence of extracellular Ca^{2+}, indicative of a Ca^{2+} influx through the membrane.

Peptide-induced pores have been reported to be anionic, cationic or non-selective (Kourie & Shorthouse, 2000:C1063) as well as to be of different sizes (Sarvazyan, 1998:297). These peptides, by the trafficking of ions, depolarized the targeted cell (Moerman, 2002:93). Along with the academic value of this kind of information, such variables may provide a possibility in the formulation of antimicrobial agents effective against bacteria. Certain strains of bacteria have built up a resistance towards antimicrobial agents available. The use of a peptide-based antimicrobial agent may be the answer in combating these strains of bacteria (Hancock & Rozek, 2002:143).

2. Aims and objectives

The aims and objectives of the study are to:

- Conclude the ion selectivity of PP and OP1-induced pores in cardiac myocytes by using the shifts in the reversal potential as indication in response to extracellular Na^+ and intracellular K^+ and Cl^- substitutions.
- Estimate the size of the PP and OP1-induced pores in cardiac myocytes by making use of an osmotic protection assay.
- Quantify the effect of PP and OP1-induced changes of membrane potential in cardiac myocytes and neuroblastoma cell line by use of a potentiometric fluorophore and confocal microscopy.

3. Hypothesis

According to Verdonck *et al.* (2000:247), PP-induced pores are expected to be non-selective as the reversal potential (E_{rev}) was in the vicinity of 0 mV in rat dorsal root ganglion cells. OP1 has shown to have similar linear α-helical structural properties to PP. This led to the formulation of the hypothesis that PP and OP1 form non-selective transmembrane pores in the membranes of cardiac myocytes that will depolarize the cells' membranes.

The literature study to follow will cover existing information relevant to this study. It will include a brief overview of the different ion channel selective peptides found in scorpion venom. Special emphasis will be placed on the antimicrobial or pore-forming peptides with regards to their structural differences, those peptides isolated from scorpion venom and the different mechanisms described for the interaction of antimicrobial peptides with cell membranes. The ionic selectivity of peptide-induced pores and the use of the reversal potential as indicator are discussed, as well as the use of an osmotic protection assay for estimation of pore size. Finally, a description of the membrane potential and the use of potentiometric fluorophores as means of quantifying changes are reported.

Chapter 2: Literature Study

1. Scorpions

Scorpions have an infamous reputation perpetuated through folklore and are associated with wickedness and with the sign of the Zodiac (Gwee *et al.,* 2002:795). They belong to the phylum Arthropoda, subphylum Chelicerata, class Arachnida, order Scorpiones. They are represented by 16 families, 159 genera and approximately 1500 different species around the world (Polis, 1990:6).

The southern African scorpion species belong to the families Bothuridae Simon 1880, Ischnuridae Simon 1879, Scorpionidae Latreille 1802 and Buthidae Koch 1837 (Polis, 1990:6). Scorpions dangerous to humans and considered to be of medical importance belong to the family Buthidae, comprising of 81 genera and 624 species (Rein, 2003). Approximately 8 genera are considered toxic to man and include *Androctonus* Ehrenberg 1828, *Leiurus* Ehrenberg 1828, *Buthus* Leach 1815 (North Africa, Middle East, India), *Parabuthus* Pocock 1890 (southern and northern Africa), *Centruroides* (southern parts of the USA, Mexico and Central Africa), *Tityus* Koch 1836 (Trinidad, Tobago and South America), *Hottentotta* Birula 1908 and *Mesobuthus* Vachon 1950 (tropical and sub-tropical regions) (Possani *et al.,* 1999:287; Rein, 2003). Of the genus *Parabuthus*, 20 species are found in southern Africa and 8 species in northeast Africa (Prendini, 2001:16). Scorpion venom is a lethal cocktail of ion channel targeting and membrane lipid-interacting peptides that have the potential to inflict a range of effects and pathological conditions.

1.1. Scorpion venom and its toxic peptides

Scorpion venom is a complex mixture composed of a wide array of substances. It contains mucopolysaccharides, hyaluronidase, phopholipase, relative low molecular mass molecules like serotonin, histamine, protease inhibitors and histamine-releasers and a rich source of toxic polypeptides that affect ion channel function of excitable and non-excitable cells (Simard & Watt, 1990:419; Possani *et al.,* 2000:861). Only a fraction of all the scorpion species' venom has been analysed to date and only a number of Na^+-channel, K^+-channel, Cl^--channel, Ca^{2+}-channel (Possani *et al.,* 2000:861) and RYR channel selective toxins have been isolated and characterized (Valdivia & Possani, 1998:111). Antimicrobial peptides have also been isolated and purified from the venom of certain scorpion species. These peptides have the ability to integrate with mammalian and bacterial membranes and form transmembrane pores (Verdonck *et al.,* 2000:247; Moerman *et al.,* 2002:4799).

Voltage-activated Na^+-channel toxins are long single-chain peptides containing 59-76 amino acids and have a molecular mass ranging between 6.5-8 kDa (Dyason *et al.*, 2002:770). The secondary structural arrangement comprises of 2 or 3 strands of antiparallel β-sheets and usually a stretch of α–helix interlinked, folded and stabilized by 4 disulphide bridges (Simard & Watt, 1990:419; Gordon *et al.*, 1998:138; Possani *et al.*, 1999:287). Exceptions to the rules are an excitatory insect toxin from *Buthotus judaicus*, with 2 short α–helix segments (Possani *et al.*, 1999:290) and birtoxin (Inceoglu *et al.*, 2001:5407). Inceoglu *et al.* (2005:727) recently reported 3 novel members to the birtoxin family, namely dortoxin, bestoxin and altitoxin. These 3 peptides are long chain peptides, a characteristic of voltage-activated Na^+-channel peptides, but have only 3 disulphide bridges. There are 2 groups of Na^+-channel selective toxins, namely α- and β-toxins, which bind to receptor site 3 and 4 respectively. The binding of such a peptide to receptor site 3 causes a delayed inactivation process and the binding to receptor site 4 shifts the threshold of activation to hyperpolarized membrane potentials (Gordon *et al.*, 1998:131; Cestele & Catterall, 2000:883).

The voltage-activated and calcium-activated K^+-channel selective toxins are short chain toxins of 31-39 amino acid residues and molecular mass of 3-4.5 kDa. Although their primary structures show highly variable amino acids sequences, they all share the same general three-dimensional structure consisting of a short α-helix and a 3 stranded β-sheet structure, stabilized mainly by 3 disulphide bridges. These toxins bind to a receptor site associated with the pore of the K^+ channel and block the outward flow of K^+ (Tytgat *et al.*, 1999:444).

Voltage-activated Ca^{2+}-channel toxins have also been isolated from the venom of scorpions. Chuang *et al.* (1998:668) initially identified a 63 amino acid residue kurtoxin in the venom of the scorpion *P. transvaalicus* Purcell 1899. A 62 amino acid residue kurtoxin-like peptide I and a 63 amino acid residue kurtoxin-like peptide II, were isolated from *P. granulatus*. These peptides have molecular masses of 7244.2 and 7386 Da, respectively. These toxins have 4 disulphide bridges that stabilize their three-dimensional structure (Olamendi-Portugal *et al.*, 2002:565). Kurtoxins bind to a receptor site in association with the pore of the channel acting as a Ca^{2+}-channel antagonist (Chuang *et al.*, 1998:668; Sidach & Mintz, 2002:2024).

Cl^--channel toxins are considered short chain toxins (like K^+-channel toxins) and are composed of 29-41 amino acid residues and stabilized by 3-4 disulphide bonds (Possani *et al.*, 2000:865). It is proposed that the secondary structure of the well known 36 amino acid residue chlorotoxin (molecular mass of 4070 Da), isolated from the venom of *L. quinquestriatus quinquestriatus* and is homologous with the insectotoxin $BeIT_5A$ containing 2 anti-parallel β-sheets and a α-helix. Chlorotoxin inhibits the function of the Cl^--channel (Debin *et al.*, 1993:C364).

The RYR targeting $IpTx_a$ and $IpTx_i$ constitutes a class of scorpion toxins targeted against intracellular ion channels. The agonistic $IpTx_a$ consists of 33 amino acid residues with a molecular mass of 3765 Da. Six cysteine residues play an important role in the three-dimensional structure of the toxin and the central structural elements are a 3 stranded β-sheet and a long loop connecting strands II and III. The antagonistic $IpTx_i$ of 15000 Da is comprised of a large PLA_2 subunit (104 amino acid residues) and a second smaller subunit (27 amino acid residues). Eight cysteine residues, resulting in 4 disulphide bridges are found in the PLA_2 subunit and a disulphide bridge links the large and the small subunits together (Valdivia & Possani, 1998:111).

A more recent class of peptides namely the antimicrobial peptides (also referred to as pore-forming and membrane disrupting peptides) is a class of peptides that targets the membranes of cells (Verdonck *et al.*, 2000:253; Torres-Larios *et al.*, 2000:5028). This class will be discussed in length later in the chapter.

1.2. Scorpion toxins cause cell death and pathological conditions

The cocktail of peptides injected into prey or predator by the scorpion contains a number of neurotoxic peptides (Simard & Watt, 1990:419; Possani *et al.*, 2000:861). Since the intact nerve appears to be relatively resistant to the action of the venom, the nerve terminals are likely the primary sites of venom action (Ismail, 1995:829). It is possible that the venom, through delaying of inactivation (α-toxins) and/or enhancing activation (β-toxins) of Na^+-channels (Müller, 1993:407), or blockage of the voltage-dependant and Ca^{2+}-dependant K^+-channels (Ismail, 1995:829), would lead to constant depolarization and a tendency to fire spontaneously and repetitively (Müller, 1993:407; Ismail, 1995:829). The toxin-induced potentiation of the duration of the action potential causes an over stimulation of the sympathetic (adrenergic) and/or parasympathetic (cholinergic) nerve endings and the release of the respective neurotransmitters, noradrenaline, adrenaline and acetylcholine (Müller, 1993:407; Bergman, 1997:167).

Researchers agree that the predominant adrenergic stimulation contributes to the cardiovascular effects of envenomation such as tachycardia, cardiac dysrhythmia and hypertension (Müller, 1993:407; Ismail, 1995:828). Generally, the hypertensive effect is so pronounced and long lasting that it is considered a major factor responsible for the development of the venom-induced cardiac failure and pulmonary edema (Ismail, 1995:828). Cholinergic stimulation causes a reduction in heart rate and a diminished venous return. Although this is proposed to act as protection against pulmonary oedema, there is no protective mechanism to counteract the reduction in heart rate and

venous return. The result would be sudden cardiac arrest or sudden acute hypotension leading to rapid death (Bergman, 1997:168). The neurotoxic peptides can have a direct cardiac stimulant affect that influences the intracellular Ca^{2+} concentrations and, therefore, the contractility of the cardiomyocytes, leading to hypercontraction and cell death (Teixeira *et al.*, 2001:708).

Symptoms include hyperirritability, focal and generalized seizures, hemiplegia, hyper and hypothermia, agitation and decreased levels of consciousness and convulsions have also been reported (Ismail, 1995:840).

Many hypotheses exist in order to explain the manner in which membrane disrupting / antimicrobial peptides kill microbes and/or eukaryotic cells. It is proposed that (i) a net movement of ions causes a fatal depolarization, (ii) creation of pores cause cellular content to leak out, (iii) the activation of deadly processes such as induction of hydrolases that degrade the cell membrane i.e. peptidoglycan autolysis, (iv) the scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbances of the membrane function and (v) the damaging of critical intracellular targets after internalization of the peptide (Tossi *et al.*, 2000:10; Zasloff, 2002:391).

2. Antimicrobial peptides

Biological membranes provide not only an isolated environment for individual cells of the body but also, for single-cell microbes, a barrier protecting them from hostile surroundings. It is not surprising that disrupting biological membranes is an effective way of injuring and eventually killing cells. Proteins or peptides that insert into membranes accomplish disruption and form pores, making the membranes permeable to ions and, in some case, larger cell organelles (Ojicius *et al.*, 1998:44). Membrane disrupting peptides are found throughout nature in organisms as diverse as plants, insects, fish, molluscs and mammals (Ojicius *et al.*, 1998:44). Such peptides have also been purified from the venom of scorpions (Verdonck *et al.*, 2000:253; Torres-Larios *et al.*, 2000:5023). The 2 common and functionally important structural requirements are a net cationicity that facilitates interaction with negatively charged membrane lipids and the ability to assume amphipathic structures that permit incorporation into membranes (Tossi *et al.*, 2000:5). The secondary structures of the cationic peptides categorize these peptides into 4 heterogeneous groups (Epand & Vogel, 1999:13; Kourie & Shorthouse, 2000:C1064; Hancock, 2001:156).

2.1. Cysteine-containing peptides with 1 disulphide bridge

Two cysteine residues are found in the primary structure of these hairpin-like antimicrobial peptides and the formation of a disulphide bridge is obtained (Vizioli & Salzet; 2002:495). Examples of such peptides are the 21 amino acid residue thanatin (cysteine 11 and cysteine 18), isolated from the bug *Podisus maculiventris* and the 24 amino acid residue brevinin 1 and 1E (cysteine 18 and cysteine 24) and the 33 amino acid residue brevinin 2 and 2E (cysteine 27 and cysteine 33) from the frog *Rana brevipoda*. The core of thanatin is a well-defined 2-stranded β-sheet structure slightly twisted and maintained by the single disulphide bridge, while the N-terminus corresponds to a long extended and poorly defined arm. This antiparallel 2-stranded β-sheet structure assimilated to a hairpin-like β-sheet structure is also found in protegrins isolated from porcine leukocytes and tachyplesins isolated from the hemocytes of horseshoe crabs (Bulet *et al.*, 1999:335).

2.2. Cysteine-containing peptides with 2 or more disulphide bridges

A well-defined group of peptides that conform to this structure are the defensins that are found in mammals, insects and plants (Vizioli & Salzet, 2002:495). Mammalian defensins are β-sheet peptides with between 29 and 40 amino acid residues and 3 intramolecular disulphide bridges (6 cysteine residues). Mammalian defensins do not form α-helices and have a region of antiparallel β-sheets (Kourie & Shorthouse, 2000:C1067). The insect defensins are peptides with 36 to 46 amino acid residues and 6 cysteine/3 disulphide bridge pattern (Bulet *et al.*, 1999:330). These defensins have a conserved structure, consisting of a N-terminal loop, a α-helical domain and a C-terminal composed of an antiparallel β-sheet (Cociancich *et al.*, 1993:17; Kourie and Shorthouse, 2000:C1067). The loop is linked by 1 of the disulphide bridges to the first strand of the β-sheet, whereas the α-helix is stabilized via the 2 other bridges to the second strand of the β-sheet. In contrast, mammalian defensins consist of β-sheets and lack a α-helix (Cociancich *et al.*, 1993:17). Hemolymph of the scorpion *Leiurus quiniquestriatus* was shown to contain a small cationic antibacterial peptide with high sequence to insect defensins. Androctonin, buthinin and a defensin-like peptide, having 25, 34 and 37 amino acid residues respectively, have been isolated from hemolymph of the scorpion *Androctonus australis*. Androctonin and buthinin possess 2 and 3 disulphide bridges, respectively (Ehret-Sabatier *et al.*, 1996:29537).

2.3. Peptides with 1 or 2 amino acids over-represented

This class of cationic peptides is enriched with a large number of proline or glycine residues (insects) and histamine or tyrosine residues (mammals) in their primary structure (Hancock, 2002:157). Drosocin (fruit fly), diptericins (Dipterans), histatins (humans) and indolicidin (cattle) are examples of proline, glycine, histamine and tyrosine-rich antibacterial peptides, respectively (Vizioli & Salzet, 2002:494).

2.4. Linear α-helical peptides

One of the larger and better-studied classes of antimicrobial peptides is those that form cationic amphipathic α-helices. Of the earliest studied groups are the cecropins from the hemolymph of the silk moth, *Hyalophora cecropia* (Zasloff, 2002:389; Vizioli & Salzet, 2002:495) and the intestines of a pig (cecropin P1) (Duclohier, 1994:183) and magainins from the skin secretions of the clawed frog (Epand & Vogel, 1999:13; Kourie & Shorthouse, 2000:C1065). Cecropins are a family of 3-4 kDa linear amphipathic peptides devoid of cysteine residues and containing 2 α-helical segments (a strongly basic N-terminal domain and a long hydrophobic C-terminal helix) linked by a short hinge (Zasloff, 2002:389). Magainin 1 and 2, from the clawed frog *Xenopus laevis*, contains 23 amino acid residues, is positively charged and contains 3 lysine residues distributed along the length of the molecule and conforms to a α-helical structure in a lipid environment (Ducohlier, 1994:176; Kourie & Shorthouse, 2000:C1065). Dermaseptin, another peptide isolated from the skin of the frog *Phyllomedusa sauvagii*, is a 34-residue peptide also shown to exhibit 77% α-helical structure (residues 1-27) in hydrophobic conditions (Mor *et al.*, 1991:8824). The cecropins, magainins and dermaseptin have been shown to inhibit the growth of bacteria, fungi and protozoa actively (Vizioli & Salzet, 2002:495).

Melittin, a 26-residue cationic peptide from the venom of the European honeybee, *Apis mellifera*, has a bent α-helical structure due to the presence of a proline residue at position 14. The peptides have 3 lysine and 2 arginine residues and have a net charge of +6 (Bechinger, 1997:202).

Scorpion venom has also been shown to contain peptides belonging to a unique cysteine-free group of peptides which contains cysteine amino acids in their structure (Torrs-Larios *et al.*, 2000:5028; Corzo *et al.*, 2001:39; Moerman, 2002:47). The origin, structure, amino acid sequences and functions are discussed.

3. Antimicrobial peptides from scorpion venom

Most of the effort in the discovery of new peptides in scorpion venom has been focused on ion channel toxins. However, in the past few years, 16 peptides of unique structure and function with and without cysteine residues have been isolated (Conde *et al.*, 2000:166; Zhu *et al.*, 2000:57). Parabutoporin (PP), from the venom of *Parabuthus schlechteri* (Verdonck *et al.*, 2000:253) and hadrurin, from the venom of the scorpion *Hadrurus aztecus* (Torres-Larios *et al.*, 2000:5023) were the first cysteine-free peptides purified and characterized. Fourteen of these peptides have no cysteine residues in their amino acid sequence (see Table 1) and include a common sequence of $S\,x_3\,K\,x\,W\,x\,S\,x_5\,L$ in 6 peptides and $G\,x_2\,W\,x_2\,I\,K\,S$ in 5 of the peptides ("x" represents uncommon amino acids) (Moerman, 2002:59). The 14 linear α-helical and 2 cysteine-rich antimicrobial peptides are discussed in detail.

3.1. Linear α-helical peptides

3.1.1. Parabutoporin from *Parabuthus schlechteri*

This linear α-helical peptide forms part of the complex mixture of *P. schlechteri* Purcell crude venom. PP has a molecular mass of 5030 Da and its primary sequence can be observed in Table 1. The most remarkable characteristic is the high lysine content (11) and the amount of charged residues (17 in total). The positive (11 lysine and 1 arginine) and negative (1 aspartate and 4 glutamate) charges are located at opposite ends of the molecule (Verdonck *et al.*, 2000:253). The peptide conforms to a α-helical secondary structure in the presence of the secondary structural-promoting environment (Willems *et al.*, 2002:1681). The helix wheel projection also indicates an amphipathic α-helix character for a majority of the peptide that stretches across the membrane (residue 11-35), with the polar hydrophilic and apolar hydrophobic amino acid side chains positioned on opposite sides of the helix (Verdonck *et al.*, 2000:253).

Activity of PP has been investigated on a variety of cell types (Verdonck *et al.*, 2000:253; Willems *et al.*, 2002:1681; Moerman *et al.*, 2002:4799; Moerman *et al.*, 2003:90). The cationic, amphipathic α-helix structure allows for easy interaction with lipopolysaccharides of eukaryotic cells and, therefore, PP was initially characterized as a pore-forming peptide. The existence of leak currents originating in rat dorsal root ganglion cells (Verdonck *et al.*, 2000:254) and cardiac myocytes (Du Plessis, 1999:65) is indicative of this property. The structure also allows for interaction with the outer membranes of bacteria, thus acting as a novel class of antimicrobials working at micromolar concentrations (Moerman *et al.*, 2002:4805; Willems *et al.*, 2002:1683).

PP is most active in inhibiting the growth of Gram-negative bacteria (MIC 1.6-6.3 μM) over Gram-positive bacteria (6.3->50 μM) (Moerman *et al.*, 2002:4805). The large polar portion of the helix, high positive charged and extended angle subtended by the positively charged residues could probably explain the profound activity towards Gram-negative over Gram-positive bacteria (Moerman *et al.*, 2002:4808).

PP has also been found to have an effect on the intracellular Ca^{2+} concentration of granulocytes in the presence and absence of extracellular Ca^{2+}, indicating the pore-formation as well as the involvement of G-proteins in the release of Ca^{2+} from intracellular stores respectively (Moerman *et al.*, 2003:95). This peptide has also been reported to inhibit (by suppression of NADPH oxidase via a Rac activation pathway) and activate human granulocytes (by stimulating exocytosis and chemotaxis) (Willems *et al.*, 2002:1683).

3.1.2. Opistoporin 1 and 2 from *Opistophthalmus carinatus*

Two 44 amino acid residue peptides, namely opistoporin 1 (OP1) and 2 have been isolated from the southern African scorpion specie *O. carinatus* Peters, 1861 (family Scorpionidae Latreille 1802) with molecular masses of 4833.6 and 4870 Da, respectively. The only difference being different amino acid residues at position 34 (leucine in opistoporin 1 and phenylalanine in opistoporin 2) (Table 1) (Moerman *et al.*, 2002:4802). These peptides contain 12 charged residues (8 lysine, 3 glutamate and 1 aspartate) and have a +4 net charge. No cysteine residues were found in the amino acid sequence. Both have α-helical secondary structure in a phospholipid-mimicking environment. Opistoporins contain 2 α-helical domains (residue 3-14 and 20-39) separated by a random coiled region (WNSEP). It is also found that the OP1 possesses hydrophobic and hydrophilic residues on opposite sides of its helical wheel diagram (residues 20-37), indicating an amphipathic nature (Moerman *et al.*, 2002:4803-4804).

OP1 has a variety of functions including pore-formation, antimicrobial activity and interaction with G-proteins (Moerman *et al.*, 2002:4804; Willems *et al.*, 2002:1679; Moerman *et al.*, 2003:90). OP1 tends to exhibit larger activity towards Gram-negative (MIC 1.6-50 μM) over Gram-positive bacteria (MIC > 50 μM). This peptide allows for an increase in intracellular Ca^{2+} concentration in the presence and absence of extracellular Ca^{2+} (Moerman *et al.*, 2003:92). This is indicative of pore-formation and the involvement of G-proteins, respectively (Moerman *et al.*, 2003:92). OP1, just like PP, has also been proven to inhibit NADPH oxidase formation in granulocytes (Willems *et al.*, 2002:1683).

Table 1: Amino acid sequencing of (A) linear α-helical and (B) cysteine-rich antimicrobial peptides isolated from scorpion venom

(A) Linear, α-helical peptides have the common sequencing of *S* x_3 *K x W x S* x_5 *L* (#) and/or *G* x_2 *W* x_2 *I K S* (▲). (B) Cysteine residues (red) allowing for the conformational folding and β-sheet formation of the peptide.

A	Ref	Amino acid sequencing	
parabutoporin	1; 2	FKLGSFLKKA$_{10}$WKSKLAKKLR$_{20}$AKGKEMLKDY$_{30}$AKGLLEGGSE$_{40}$EVPGQ #	5030 Da
opistoporin 1	2	GKVWDWIKST$_{10}$AKKLWNSEPV$_{20}$KELKNTALNA$_{30}$AKNLVAEKIG$_{40}$ATPS # ▲	4833.6 Da
opistoporin 2	2	GKVWDWIKST$_{10}$AKKLWNSEPV$_{20}$KELKNTALNA$_{30}$AKNFVAEKIG$_{40}$ATPS # ▲	4870 Da
hadrurin	3	GILDTIKSIA$_{10}$SKVWNSKTVQ$_{20}$DLKRKGINWV$_{30}$ANKLGVSPQA$_{40}$A #	4435.6 Da
pandinin 1	4	GKVWDWIKSA$_{10}$AKKIWSSEPV$_{20}$SQLKGQVLNA$_{30}$AKNYVAEKIG$_{40}$ATPT # ▲	4799.2 Da
pandinin 2	4	FWGALAKGAL$_{10}$KLIPSLFSSF$_{20}$SKKD	2612.6 Da
IsCT	5	ILGKIWEGIK$_{10}$SLF ▲	1501.9 Da
IsCT2	6	IFGAIWNGIK$_{10}$SLF ▲	1463.92 Da
BmKbpp	7	FRFGSFLKKV$_{10}$WKSKLAKKLR$_{20}$SKGKQLLKDY$_{30}$ANKVLNGPEE$_{40}$EAAAPAERRR$_{50}$ #	Unknown
BmKn1	10	FIGAVAGLLS$_{10}$KIF	Unknown
BmKn2	10	FIGAIARLLS$_{10}$KIF	Unknown
BmKa1	10	GESEENEEGS$_{10}$NESGKSTEAK$_{20}$NTDASVDNED$_{30}$SDIDGDSD	Unknown
BmKa2	10	YPASMDNSDD$_{10}$ALEELDNLDL$_{20}$DDYFDLEPAD$_{30}$FVLLDMWANM$_{40}$LESSDFDDME	Unknown
BmKb1	10	FLFSLIPSAI$_{10}$SGLISAFK	Unknown
B			
scorpine	8	GWINEEKIQK$_{10}$KIDERMGNTV$_{20}$LGGMAKAIVH$_{30}$KMAKNEFQCM$_{40}$ANMDMLGNCE$_{50}$KHCQTSGEKG$_{60}$YCHGTKCKCG$_{70}$TPLSY	8350 Da
BmTXKS2	9	DKYCSENPLD$_{10}$CNEHCLKTKN$_{20}$QIGICHGANG$_{30}$NEKCSCMES	Unknown

1 - Verdonck *et al.*, 2000:247; 2 - Moerman *et al.*, 2002:4799; 3 - Torres-Larios *et al.*, 2000:5028; 4 - Corzo *et al.*, 2001:38; 5 - Dai *et al.*, 2001:823; 6 - Dai *et al.*, 2002:1519; 7 - Zeng *et al.*, 2000:208; 8 - Conde *et al.*, 2000:165; 9 - Zhu *et al.*, 2000:57; 10 - Zeng *et al.*, 2004:143.

3.1.3. Hadrurin from *Hadrurus aztecus*

The Mexican scorpion specie *H. aztecus* contains the peptide hadrurin in its crude venom and it accounts for ~1.7% of the total protein of the venom. It has a molecular mass of 4435.6 Da and 41 amino acid residues in it's primary sequence (Table 1). There are 7 basic amino acid residues, 3 of which are grouped as a triplet of sequence lysine-arginine-lysine. Amino acid residues 1-11 and 18-41 indicate α-helical structures with the hydrophobic and hydrophilic residues on opposite sides of the helix (Torrs-Larios *et al.*, 2000:5028-5029).

Hadrurin inhibited Gram-positive (MIC < 10 μM) and Gram-negative bacteria (MIC > 40 μM). It is clear that hadrurin is more active towards Gram-negative bacteria (Torres-Larios *et al.*, 2000:5026). The peptide has cytolytic effects on erythrocytes, showing a HD_{80} value of 20 μM (Torres-Larios *et al.*, 2000:5028).

3.1.4. Pandinin 1 and 2 from *Pandinus imperator*

P. imperator crude venom was screened and 2 antimicrobial peptides, namely the 44 and 24 residued pandinin 1 (4799.2 Da) and pandinin 2 (2612.6 Da) respectively, were identified and isolated (Table 1). In aqueous solutions these peptides have an unordered structure, but a α-helical structure is obtained in membrane-mimicking environment (PBS and DPC). Pandinin 1 was shown to contain 2 α-helical regions (residues 3-14 and 20-39), separated by a random coil region (WSSEP) including a proline residue at position 19. The α-helix of both peptides is amphipathic, with hydrophobic and hydrophilic residues on opposite sides of the helices (Corzo *et al.*, 2001:39).

Pandinin 1 and 2 showed growth inhibition of Gram-positive and Gram-negative bacteria. Hemolytic activity was obtained in the presence of pandinin 2 (Corzo *et al.*, 2001:39).

3.1.5. IsCT and IsCT2 from *Opisthacanthus madagascariensis*

Two peptides, IsCT and IsCT2, were characterized from the scorpion specie *O. madagascariensis*. The peptides have molecular masses of 1501.9 and 1463.9 Da, respectively. IsCT is composed of 13 amino acid residues and enriched with hydrophobic (3 isoleucine, 2 leucine) and basic amino acids (2 lysine). IsCT2 has 78% homology with IsCT and also consists of 13 amino acid residues, but differing only by the replacement of lysine at position 4 with arginine and glutamate at position 7 with asparagine. Both peptides have an amphipathic α-helical secondary structure in the presence of secondary structural promoting solution (60% TFE) and the arrangement of the hydrophobic and hydrophilic residues are on opposite sides of the α-helical structure (Dai *et al.*, 2001:821; Dai *et al.*,

2002:1516). These peptides have low homology with other scorpion peptides and high homology with cytotoxic peptides from wasp venom (Dai *et al.*, 2001:823).

Growth inhibition of Gram-positive (MIC of 1-25 μg/ml) and Gram-negative (MIC of 5-200 μg/ml) was observed by both peptides, although a slight preference towards Gram-positive inhibition was seen (Dai *et al.*, 2002:1517). IsCT and IsCT2 are both hemolytic towards sheep erythrocytes, having a HD_{50} of 50-75 μM (Dai *et al.*, 2001:822).

3.1.6. BmKbpp, BmKn1 and 2, BmKa1 and 2, BmKb1 from *Buthus martensii* Karsch

A 50 amino acid residue peptide, BmKbpp, is found in the venom of the Asian scorpion specie *B. martensii* Karsch and is similar to the amino acid sequence of a bradykinin-potentiating peptide (peptide K-12) from the scorpion *B. occitanus* (Zeng *et al.*, 2000:209). The amino acid sequencing has 61.7% homology with PP (Moerman, 2002:58). Literature concerning this peptide is limited and no helical wheel or circular dichroism spectra exist at this stage, but it is predicted that this peptide will be highly α-helical and linear in nature because of the absence of cysteine residues (Moerman, 2002:62). BmKn1 and 2, BmKa1 and 2 and BmKb1 have been isolated from the same scorpion venom. These peptides are presented in Table 1. No cysteine residues are found in the amino acid sequence giving indication to a possible α-helical primary structure (Zeng *et al.*, 2004:143).

3.2. Cysteine-rich antimicrobial peptides

3.2.1. Scorpine from *Pandinus imperator*

A 75 amino acid residue peptide isolated from the scorpion specie *P. imperator* inhibits the growth of ookinete (ED_{50} 0.7 μM) and gamete (ED_{50} 10 μM) stages of the malaria inducing parasite *Plasmodium berghei*. This antimicrobial peptide has a molecular mass of 8350 Da and is stabilized by 3 disulphide bonds. Scorpine's amino acid sequence (Table 1) is unique and the N-terminal and C-terminal are similar to some cecropins and defensins, respectively. Together with scorpine's anti-malaria properties, the peptide inhibits the growth of Gram-positive and Gram-negative bacteria, indicating more activity against *Klebsiella pneumoniae* (MIC of 0.1 μM) than *Bacillus subtilis* (MIC of 10 μM) (Conde *et al.*, 2000:166).

3.2.2. BmTXKS2 from *Buthus martensii* Karsch

From cDNA coding an insect defensin-like peptide has been reported in the venom of *B. martensii* Karsch. The mature peptide has an amino acid sequence of 39 residues containing 6 cysteine

residues. Together with a conserved glysine residue, it is similar to that of LqDef, a defensin found in the hemolymph of the scorpion specie *L. quinquestriatus* and is stabilized by a α-β-motif (Zhu *et al.*, 2000:57).

As stated previously, these peptides interact with the membranes of various cell types. Various factors contribute to the interaction with mammalian and/or bacterial membranes and 3 distinct mechanisms of interaction have been proposed.

4. Interaction of linear α-helical peptides with membranes

Biological membranes contain a large variety of lipids. One property of these membranes that has been associated with antimicrobial specificity is their negative charge. Several antimicrobial peptides are cationic and preferentially bind to anionic lipids (Tossi *et al.*, 2000:10; Hancock, 2001:159).

The membrane of Gram-negative and Gram-positive bacteria is negatively charged due to the anion rich lipopolysaccharides and teichoic and teichuronic acids in the peptidoglycan layer in the outer most layer of the membrane respectively (Tossi *et al.*, 2000:10). Most of the anionic lipids of mammalian membranes are sequestered on the cytoplasmic side of the membrane and this can provide a potential mechanism for microbial specificity (Epand & Vogel, 1999:18). These cationic α-helical peptides are more effective in inducing leakage in liposomes of phosphatidylglycerol, a lipid which is found in high abundance in microbial membranes, than in liposomes of phosphatidylserine, a major anionic lipid of mammalian membranes (Epand & Vogel, 1999:18).

The formation of transmembrane pores is a dynamic process that depends on a variety of factors, the electrostatic and hydrophobic interactions and the composition of the phospholipid head groups and the fatty acid chains, peptide-to-lipid ratio (Bechinger, 1999:157; Lee *et al.*, 2004:3591). Bechinger (1999:157) further describes that the activity of membrane-interacting peptides as being a multi step process of accession to the membrane, bilayer association, insertion and pore formation: water soluble ↔ surface accessible ↔ surface association ↔ bilayer inserted ↔ openings. A critical peptide-to-lipid concentration is also required for the formation of transmembrane pores (Lee *et al.*, 2004:3591), but the stability of the pore may depend on the degree of electrostatic and/or hydrophobic interactions. The following models are proposed for the interaction of cationic α-helical antimicrobial peptides with cell membranes.

4.1. 'Barrel-stave' pores

The 'barrel-stave' mechanism (C in Fig. 1) is described for the action of alamethicin, a peptide from the fungus *Trichoderma viride* (Bechinger, 1997:203; Tossi *et al.*, 2000:10). The formation of transmembrane pores by bundles of amphipathic α-helices, such that their hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward, producing an aqueous pore (Shai, 1999:60). The following stages form the process of pore formation via the 'barrel-stave' model: (i) monomers bind to the membrane surface in a parallel fashion and its unordered structure changes to an α-helical structure, (ii) monomers recognize each other in the membrane-bound state already at low surface density of bound peptides, (iii) helices insert perpendicularly into the hydrophobic core of the membrane and (iv) progressive recruitment of additional monomers occurs to increase pore size (Shai, 1995:460; Shai, 1999:60; Tossi *et al.*, 2000:11).

4.2. 'Toroidal'/'worm-hole' pores

This mechanism of transmembrane pore formation is well described for magainins (Ludtke *et al.*, 1996:13723). This model is similar to the 'barrel-stave' model but differs in that the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (Matsuzaki *et al.*, 1995:3427). In forming such a pore the lipid monolayer bends continuously from the top to the bottom in the fashion of a toroidal hole, so that the pore is lined by both the peptides (hydrophilic residues) and the lipid head groups (B in Fig. 1) (Ludtke *et al.*, 1996:13727; Yang *et al.*, 2001:1476).

4.3. 'Carpet' pores

The 'carpet' model (D in Fig. 1) was proposed for the first time to describe the mode of action of dermaseptin S, an antimicrobial peptide isolated from the skin secretions of the frog genus *Phyllomedusa* (Shai, 1999:60; Tossi *et al.*, 2000:9). The peptides conforming to this type of peptide-lipid interaction are in contact with the phospholipids head group throughout the entire process of membrane permeation. Membrane permeation occurs only if there is a high local concentration of membrane-bound peptides and this can occur either when the entire membrane surface is covered with peptide monomers, or alternatively, after there is an association between membrane-bound peptides, forming a localized 'carpet' (Shai, 1999:60; Tossi *et al.*, 2000:10). A peptide that permeates the membrane via this mechanism does not necessarily require the adoption of a specific

structure upon its binding to the membrane. Initial interaction with the negatively charged target membrane is electrostatically driven and, therefore, peptides are positively charged (Shai, 1995:461; Shai, 1999:60). Firstly, monomers bind to the phospholipids head groups in the membrane. Secondly, the monomers align themselves on the surface of the membrane so that their hydrophilic surface is facing the phospholipids head groups or water molecule. Thirdly, the molecules rotate leading to reorientation of the hydrophobic residues towards the hydrophobic core of the membrane, and fourthly, the molecule disintegrates the membrane by disrupting the bilayer curvature (Shai, 1999:60). An earlier step before the collapse of the membrane packing may include the formation of transient holes in the membrane, which enable the passage of low molecule weight molecules prior to complete lysis (Shai, 1999:60).

4.4. Shai-Matsuzaki-Huang model

Zasloff (2002:391) proposes a model of antimicrobial peptide interaction with lipid layers. It incorporates the 3 above-mentioned models and includes the interaction of the peptide with the membrane, disruption of the membrane, diffusion to the intracellular fluid and targeting the intracellular targets by the peptides.

It is clear from the models that transmembrane pores are formed (Fig. 1). These pores are unique in that they differ in their ion-selectivity, meaning that pores are cation, anion or non-selective (Hille, 2002:364; Gincel *et al.*, 2004:721).

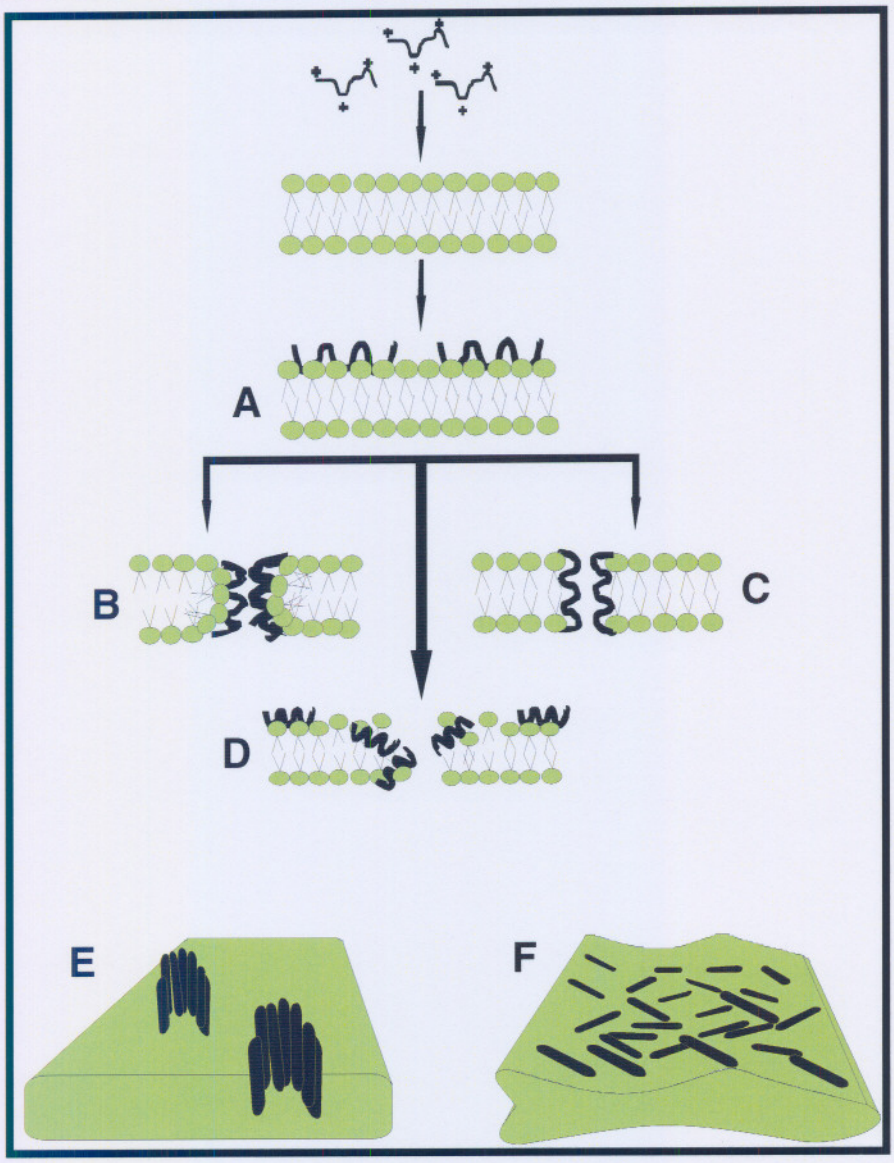

Figure 1: Interaction of cationic α-helical antimicrobial peptides with cell membranes.
(A) Unordered peptides interact with cell membrane and adopt an α-helical structure. The peptides can interact with the lipid heads and `toroidal` (B) or `carpet` (D) pores may form. The peptides may not bind with the lipid heads and span the membrane forming `barrel-stave` pores (C). In the formation of `toroidal` and `barrel-stave` pores the peptide monomers bind together and form transmembrane pores (E) whereas the `carpet` pores show destabilization of the membrane structure (F). (Adapted from Shai, 1995:460; Tossi *et al.*, 2000:11)

5. Selectivity of peptide-induced transmembrane pores

5.1. Monovalent cation-selective gramicidin A pores

Gramicidin A (Figure 2) is an antibiotic peptide isolated from the soil bacteria *Bacillus brevis* (Killian, 1992:392). It is a linear pentadecapeptide with 15 hydrophobic alternating D and L-amino acids, with the N-terminal and C-terminal blocked by a formyl group and an ethanol amine, respectively. In the less abundant gramicidin B and C, the tryptophan at position 11 is replaced by phenylalanine or tyrosine, respectively. In 5-20% of the molecules valine at position 1 is replaced with isoleucine (Wallace, 1990:127; Killian, 1992:392).

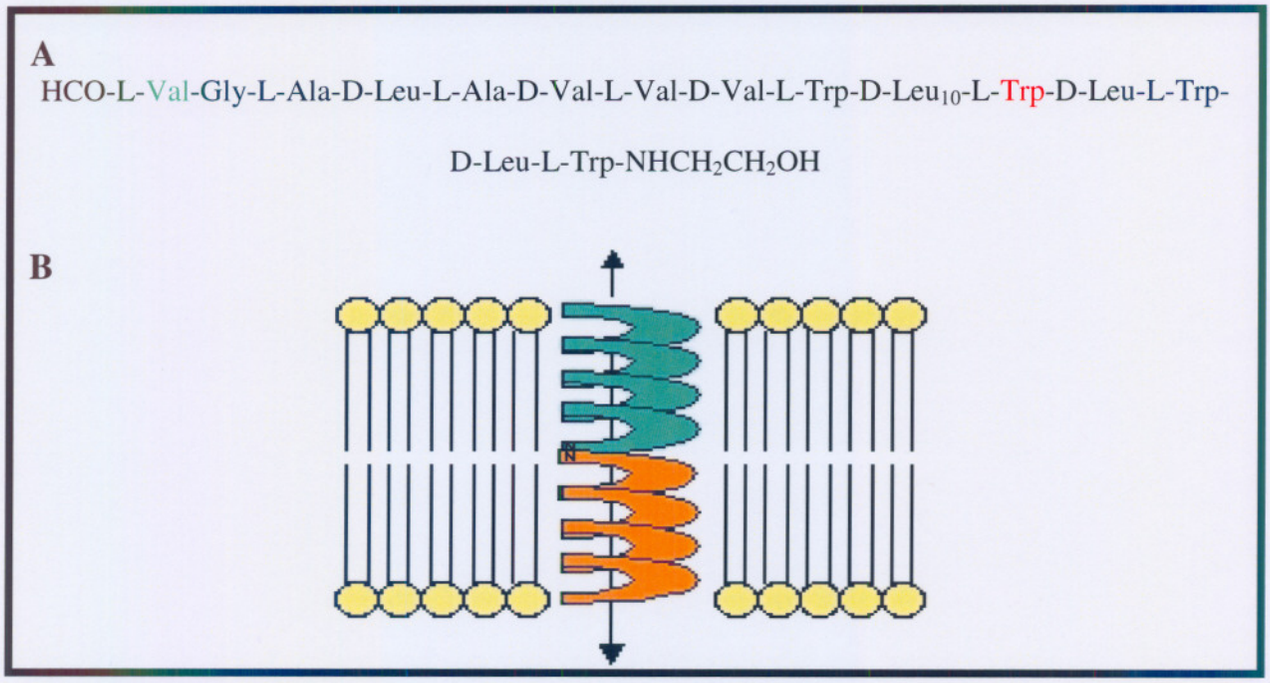

Figure 2. (A) Amino acid sequencing and (B) conformation of gramicidin A in a lipid bilayer. The green L-Val can be L-isoleusine and the red L-Trp may be L-Phe (Gramicidin B) or L-Tyr (Gramicidin C). (Adapated from Wallace, 1990:128)

Although gramicidin A is able to adopt a variety of conformations (Killian, 1992:394), 2 prominent conformations are observed. A single-stranded helical dimer configuration can be seen in Figure 2B. Each peptide is held together by twelve intramolecular hydrogen bonds. The 2 peptides bind N-terminal-to-N-terminal and are stabilized by 6 intermolecular hydrogen bonds between the formyl end groups (Hille, 2002:364; Wallace, 1990:144). A double-stranded helical dimer configuration differs in that the peptides run antiparallel to each other (Wallace, 1990:141; Killian, 1992:394). The conformation is influenced by the ion-bound state of the channel (Wallace, 1990:143). Breaking and reformation of the 28 hydrogen bonds depends on the ion to be moved through the channel/pore.

The breaking of these hydrogen bonds, leading to the disconnection of the transmembrane path, could be the reason for the opening and closing states that are seen in the single-channel registration measured in a variety of membranes (Wallace, 1990:145; Hille, 2002:3).

Gramicidin A channels are cation selective (Finkelstein & Andersen, 1981:155; Wallace, 1990:128; Hille, 2002:364). When there is a gradient of monovalent chloride salt across the bilayer membrane, the reverse potential equals the Nernst potential for the cation, showing that the permeability of the pore to Cl^- is negligible (Hille, 2002:364). The permeability sequence is $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ \geq Na^+ > Li^+$ (Myers & Haydon, 1972:319; Hille, 2002:364).

5.2. Reversal potential shift and ion selectivity

Whole-cell (Fidzinski *et al.*, 2003:35; Lang *et al.*, 2004:319) and single channel (Guinamard *et al.*, 2004:78; Nishio *et al.*, 1996:293) studies focusing on the ionic selectivity of transmembrane pores have been performed on lipid and artificial membranes. Most conclusions on ion selectivity are made from pores in artificial lipid bilayers facilitating interpretation on mechanisms of action (Kourie & Shorthouse, 2000:C1078). In such experimentation the complexity of the solutions to sustain a favourable environment does not influence or complicate the determination of ion selectivity of a peptide-induced pore.

Pores formed by natural/synthetic peptides or molecules have been reported to be either anionic (melittin and magainin I), cationic (gramicidin A, maitotoxin and halitoxin) or non-selective (amyloid β peptide) (Simmons & Schneider, 1993:133; Nishio *et al.*, 1996:293; Scott *et al.*, 2000:119; Hille, 2002:364; Gincel *et al.*, 2004:721). From reports selectivity seems to be a dynamic process and may depend on exposure time and peptide concentration (Bechinger, 1999:158; Lee *et al.*, 2004:3590).

The Nernst equation can be used as an indication of pore selectivity if only 1 ion is permeable through the pore. The Nernst equation,

$$E_{rev} = -\frac{RT}{ZF} \ln \frac{(ion^+)_e}{(ion^+)_i}$$

gives a theoretical value of the reverse potential for 1 transmembrane ion (Ashcroft, 2000:25; Hille, 2001:17). If the experimental E_{rev} differs from that of the theoretical value (Nernst potential), then more than 1 ion contributes to the peptide-induced current. This theoretical determination is more complex under experimental conditions as more than 1 transmembrane ion (with different

permeabilities) are present on either side of the membrane and contribute to the pore-induced current. In this case the Goldman-Hodgkin-Katz equation,

$$E_{rev} = -\frac{RT}{ZF}\ln\frac{(X^+)_e \cdot PX^+ \cdot (Y^+)_e \cdot PY^+ \cdot (Z^-)_i \cdot PZ^-}{(X^+)_i \cdot PX^+ \cdot (Y^+)_i \cdot PY^+ \cdot (Z^-)_e \cdot PZ^-}$$

where $(X^+)_{i/e}$, $(Y^+)_{i/e}$ and $(Z^-)_{i/e}$ indicate the intra and extracellular ion concentrations respectively and PX^+, PY^+ and PZ^- indicate the membrane's permeability of the respective ions can be used. This also proves to be a difficult task, as the permeabilities of the transmembrane ions are not known for the peptide-induced pore under scrutiny. Therefore, when natural membranes are used the shift in E_{rev}, due to the substitution of different ions, is an acceptable indicator of ion selectivity (Scott *et al.*, 2000:119; Gincel *et al.*, 2004:721).

The trafficking of ions across the membrane of cells results in a change in a number of cellular functions, including changes in membrane potential.

6. The membrane potential

The term membrane potential refers to a potential difference between the intra and extracellular environments. This potential stems from the equilibrium fluxes of ions across a membrane containing ion-selective channels (Plášek & Sigler, 1996:101).

In most eukaryotic cells, resting plasma membranes are considerably permeable to several ions, particularly to K^+, Na^+, and Cl^-, and together with the sodium-potassium pump (Guyton & Hall, 2000:54) result in a resting membrane potential of -30 to -90 mV, depending on the cell type (Lemasters *et al.*, 1999:346). The transmembrane concentration gradients of these ions, together with their respective membrane permeabilities, control the steady state transmembrane ionic fluxes, and thus the value of the membrane potential (Plášek & Sigler, 1996:102). The resting membrane potential can be theoretically calculated by use of the Goldman-Hodgkin-Katz equation as mentioned above (Plášek & Sigler, 1996:102; Aschroft, 2000:67).

The membrane potential is often measured by standard electrophysiological methods, providing the cell size permits microelectrode-membrane junction, or by use of potentiometric dyes together with digitally enhanced images taken with a confocal microscopy (Lemasters *et al.*, 1999:346; Loew *et al.*, 2002:429). The potentiometric dyes are used to indicate voltage differences across a cellular membrane.

6.1. Potentiometric fluorophores

Two classes of potentiometric dyes, namely the fast and slow response dyes, can be used to investigate various aspects of the membrane potential. The fast response dyes enable the quantification of electrical field changes in the membrane whilst the slow response dyes move across the membrane until they reach electrochemical equilibrium and allow for the quantification of the membrane potential changes (Farkas *et al.*, 1989:1053; Plášek & Sigler, 1996:102; Loew *et al.*, 2002:429).

Researchers proposed a method to measure the membrane potential changes quantitatively. A proposition that the intra and extracellular fluorescence intensities, which are proportional to the intra and extracellular dye concentration (Loew *et al.*, 2002:430), can be substituted into the Nernst equation and the membrane potential can theoretically be calculated. Often slow response dyes distribution deviates significantly from that predicted Nernst equation because of the binding to the plasma and organelle membranes and the tendency of these compounds to form aggregates when their concentrations exceed a threshold (Loew *et al.*, 2002:432). This led to the development of 2 rhodamine class dyes, tetramethylrhodamine methylester (TMRM) and tetramethylrhodamine ethylester (TMRE). These membrane-permeant cations are driven across the membrane by potential differences and they equilibrate in accordance to the Nernst equation. These 2 dyes have a directly proportional ratio of intra and extracellular dye concentration and fluorescence intensities (Lemaster *et al.*, 1999:346; Loew *et al.*, 2002:434). Therefore,

$$MP = -60\log\left(\frac{[\text{fluorophore}]_{\text{intra}}}{[\text{fluorophore}]_{\text{extra}}}\right) = -60\log\frac{\text{fluorescence}_{\text{intra}}}{\text{fluorescence}_{\text{extra}}}\,\text{mV}$$

where $[\text{fluorophore}]_i$, $[\text{fluorophore}]_e$, F_i and F_e are the concentration of intra and extracellular dye concentrations and are intra and extracellular fluorescence intensities respectively. It is important to know that the dye concentration in the above equation refers to free monomeric aqueous dye. Cell hyperpolarization and depolarization will be reflected by an increase and decrease of dye accumulation and, therefore, fluorescence respectively (Chacon *et al.*, 1994:943; Plášek & Sigler, 1996:103).

Along with a peptide's permeability ions resulting in membrane potential changes, peptide-induced pores are different in size. The use of osmotic protection assays together with morphological changes as indication of cardiac myocyte malfunctioning allows for the estimation of pore size.

7. Osmotic protection assay and cell death

Several methods have been used to assess the quality of isolated adult cardiac myocytes as well as to monitor their survival rates under different experimental conditions. The 3 most widely used assays to determine cardiac myocytes viability are: i) exclusion of trypan blue dye, ii) amount of released lactate dehydrogenase and iii) the manual counts of rod-shaped cells. Generally, good correlation exists between all 3 models, with cell morphology being the most sensitive index of cardiac myocytes injury. The typical intact cardiac myocytes is a striated, rod-shaped cell, 10-30 μm in width and 80-150 μM long. When damaged, the cell shortens and then hypercontracts irreversibly, giving the appearance of a sphere of about 30-40 μM in diameter (Sarvazyan, 1998:297). Spherical cells are indicative of malfunctioning, hypercontracted cells, and lead to cell death (Ver Donck & Borgers, 1991:H1829).

Many pore-forming peptides induce cell lysis by means of colloid osmotic shock (Tossi *et al.*, 2000:10). This type of cell death is caused by the trafficking of ions in (Na^+, Ca^{2+}, Cl^-) and out (K^+) and the movement of small cellular structures out of the cell. The interior of the cell becomes hyperosmotic and a net influx of water cause cell swelling until the membrane breaks and the intracellular content is released (Menestrina *et al.*, 1994:251). This process together with cell membrane destabilization properties of certain peptides causes cell death (Shai, 1999:60; Tossi *et al.*, 2000:10).

The addition of certain osmoticants (30 mM) to the extracellular environment of the cell can prevent cell lysis from occurring. The osmoticants ensure a hypertonic extracellular environment, whereby preventing the influx of water into the cell. This protection mechanism is only effective if the diameter of the osmotic protection molecules is larger that the lumen diameter of the peptide-induced pore in the cell membrane. By using a variety of osmotic protection molecules with different diameters one can estimate the size of the pore induced in various cell types (Macek *et al.*, 1994:207; Menestrina *et al.*, 1994:251; Sarvazyan, 1998).

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Chapter 3

(Note: Justification of the text was only used for the purpose of the dissertation)

Title

Ion selectivity of scorpion toxin-induced pores in cardiac myocytes.

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Abstract

The lytic activity of parabutoporin (PP) and opistoporin 1 (OP1) on mammalian and bacterial membranes have been described. Pore-formation and ion selectivity in cardiac myocytes was investigated by measuring the whole cell leak current by means of the patch clamp technique. Pore formation was observed as the induction of leak currents. Ion selectivity of the pores was indicated by the shift of the reversal potential (E_{rev}) upon substitution of intra and extracellular ions. Results were compared with the effect of gramicidin A (gramA). PP and OP1 induced a fluctuating leak current and indicate non-selectivity of PP-induced pores. PP and OP1-induced pores are between 1.38 nm and 1.78 nm in diameter.

Keywords

pore-forming, peptides, antimicrobial, ion selectivity, scorpion, venom.

1. Introduction

Scorpion venom is a mixture of mucopolysaccharides, hyaluronidase, phospholipase, low molecular mass molecules like serotonin and histamine releasers [22; 27] and is a rich source of peptide toxins that interact with different kinds of ion channels in eukaryotic cells. These peptides affect the functioning of Na^+ [23; 24], K^+ [32], Ca^{2+} [3] and Cl^- channels [4]. In addition to ion channel modulators, peptides with antimicrobial activity have been described in several species [34; 30].

Peptides with antimicrobial properties have been characterized into four groups according to the diversity of their structures, namely, peptides with cysteine residues stabilized by i) one or ii) two or more disulphide bridges, iii) peptides with over-represented amino acids and iv) linear α-helical peptides without cysteine residues [9; 6]. These peptides serve as defences against bacterial invasion and various mechanisms of action are proposed [9; 35]. These linear α-helical peptides have been isolated from the venom of southern African scorpion species, namely PP from *Parabuthus schlechteri* Purcell, 1899 [34] and OP1 from *Opistophthalmus carinatus* Peters, 1861 [19].

PP and OP1 are peptides of 45 and 44 amino acid residues, respectively [32; 17]. PP (0.5 μM) and OP1 (0.8 μM) influence membrane bound signal molecules (G-proteins, NADPH oxidase) and induced Ca^{2+} release from internal stores of HL-60 granulocytes [20; 37]. At such low concentrations no ion current across the plasma membrane was observed indicating that pore-forming activity was absent. At higher concentrations PP (1 μM) and OP1 (8 μM) induced Ca^{2+} influxes indicating the formation of pores in the membrane [18]. PP-induced leak currents have been observed in rat dorsal root ganglion cells [34].

Pores formed from natural or synthetic peptides have been reported to be either anionic (melittin and magainins) [12; 7], cationic (gramicidin A, halitoxin) [26; 7] or non-selective (amyloid β-peptide (25-35)) [28]. From reports selectivity seems to be a dynamic process and may depend on exposure time and peptide concentration [14; 2]. Pore formation is associated with depolarization of the membrane and dissipation of ion gradients causing osmotic swelling and lysis of cells [12]. Most conclusions on ion selectivity were made from pores in artificial lipid bilayers facilitating interpretation on mechanisms of action [12]. When natural membranes were used as the target for

pore-forming peptides the shift of the reversal potential or zero-current potential (E_{rev}) has mostly been used as an indicator for selectivity [7; 29; 26; 21; 28].

Osmotic protection assays have been used to estimate the pore size induced by hemolytic toxins in erythrocyte membranes [16]. An osmotic protection molecule smaller than the diameter of the peptide-induced pore will be able to flow through the pore together with the cell's ionic content, small compounds and water. Cell lysis will occur and lead to cell death. Osmotic protection molecules larger than the pore limits this trafficking through the pores and prevents cell death from occurring [16]. This assay in combination with the counting of rod-shaped cardiac myocytes [25] enables an estimation of the size of peptide-induced pores in this type of cell. A pathological stimulus leads to round-shaped cardiac myocytes and represents an irreversible hypercontraction, which can be regarded as an indication of malfunctioning and eventually leads to cell death [33; 15]. Osmotic protection molecules larger than a peptide-induced pore will show more rod-shaped cells indicating cell protection [6].

The pore-forming properties of PP and OP1 and ion selectivity with whole-cell voltage clamp in rat cardiac myocytes were investigated. The PP and OP1-induced pore sizes were estimated by performing osmotic protection assays.

2. Material and Methods

2.1. Peptides

PP (Swiss-Prot Accession No. P83312) and OP1 (Swiss-Prot Accession No. P83313) were chemically synthesized by Ansynth Service BV (The Netherlands) as described earlier [19]. Gramicidin A (gramA) was purchased from ICN Biomedicals, Inc. (Irvine, CA, USA).

2.2. Enzymatic isolation of rat ventricular cardiac myocytes

Ventricular cardiac myocytes were isolated from Sprague-Dawley rats (~200g) using the enzymatic dispersion method developed by Mitra and Morad [17] and revised by Tytgat [31]. 24 mg collagenase (type II, Sigma, St. Louis, MO, USA) and 4.8 mg protease (type XIV, Sigma, St. Louis, MO, USA) were used.

2.3. Electrophysiological measurements

The ionic leak currents of the isolated cardiac myocytes were measured at room temperature with the use of the patch-clamp technique in the whole cell configuration [8]. Current measurements and data acquisition were performed with a Dagan 8800 total clamp amplifier (Dagan Corporation, Minneapolis, MN, USA) which is controlled by a personal computer with pClamp version 5.5 software (Axon Instruments, Inc., Foster City, CA, USA). Pipettes with resistance of 1.5-3 MΩ were pulled from borosilicate capillaries with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA, USA). All GΩ seals were made in Tyrode solution in a static bath, followed by a 5 min internal dialysis period. Cells were then perfused with the relevant extracellular solutions for 5-6 min; whereafter perfusion was stopped before recordings started. Peptide was added with a micropipette.

All cells were clamped at a holding potential of -80 mV, hyperpolarized to -100 mV (15 ms) followed by depolarizing steps to -40 mV (30 ms), 0 mV (80 ms) and +40 mV (60 ms).

2.3. Solutions

The composition of the external solution (ES_1) was (in mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 11.6 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonicacid (HEPES), 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. With Na^+-substitution solution, NaCl was replaced with *N*-methyl-D-glucamine

chloride (NMDGCl), pH 7.4 with HCl (ES_2). ES_3 was composed of (in mM): 145 NaCl, 0.5 mM $CaCl_2$, 10 mM glucose, 0.5 $MgSO_4$, 5 mM HEPES, pH of 7.4 with HCl.

The internal solution (IS_1) was (in mM): 140 KCl, 2 $MgCl_2$, 10 HEPES, 1 $CaCl_2$, 11 EGTA, 5 Na_2-ATP, pH 7.4 with KOH. With K^+-substitution and zero-Cl^- solution the KCl (IS_1) was substituted with 140 mM CsCl (IS_2) and 145 mM Cs-aspartate (IS_3), respectively.

2.4. Osmotic protection assay

In this assay 30 mM of each of the following different sized protectants [13] were added to Tyrode solution (diameter indicated in parenthesis): ethylene glycol (EG) (0.44 nm), polyethylene glycol (PEG) 200 (0.80 nm), PEG 400 (1.12 nm), PEG 600 (1.38 nm) and PEG 1000 (1.78 nm). The viability of cardiac myocytes was determined by manual counting of rod-shaped cells in counting chambers under an Olympus IMT-2 inverted microscope (Olympus Optical Company, Ltd, Tokyo, Japan). The number of damaged round-shaped cardiac myocytes after 50 min were expressed as a percentage of the total number of cardiac myocytes counted and calculated as follows: (number of rod-shaped cardiac myocytes at time 0) – (number of rod-shaped cardiac myocytes at time 50 min) / (number of rod-shaped cardiac myocytes at time 0) X 100.

2.5. Processing of data and statistical analysis

For off-line data analysis, Clampfit version 6.0.5 software (Axon Instruments Inc., © 1984-1993) was used. Results were expressed as the mean ± SEM. The significance of the differences was expressed using Student's paired and unpaired t-test in Origin, version 5.0 (Microcal Software Inc., © 1991-1997). In all analysis values of $p < 0.05$ (95% probability of occurrence) were considered statistically significant.

3. Results

3.1. Pore-formation in rat cardiac myocytes

Fig. 1A shows superimposed traces of PP-induced leak currents in rat cardiac myocytes. In control conditions (a in Fig. 1A) a normal, fast inactivating Na^+ current (-40 mV) and an inward Ca^{2+} current (0 mV) is observed. The addition of 1μM PP evokes an inward leak current at negative test potentials and an outward leak current at the positive test potential (e in Fig. 1A), with diminished inward Na^+ and Ca^{2+} currents. At corresponding concentrations of OP1 and gramA, leak currents were not always induced even after 10-12 min. However, by gradually increasing the concentrations, similar leak currents to PP were observed. This indicates a threshold concentration for leak current development. Leak currents as in Fig. 1A were observed in the presence of OP1 and gramA within a concentration range of 1-4μM and 1-2μM, respectively. Although the above mentioned effect was reproducible for PP and OP1, the time taken to elicit an effect as well as the time to maximum effect (morphological change from rod-shaped to round-shaped cardiac myocytes or loss of capacitance in raw data) varied between individual experiments. The effects of PP and OP1 were observed in a time period of 12-18 min and 10-14 min, respectively. These differences in time-to-effect were inversely proportional to the peptide concentration.

In addition to the variability in onset of the effect and threshold concentration for leak current induction, fluctuations in leak current in the continuous presence of the peptides were frequently observed. This is illustrated in Figure 1B for 1μM PP. In this figure the holding current at -80 mV has been plotted as a function of time. In 80% of the experiments for PP and OP1 this type of time course was observed with partial recoveries of the PP (Fig. 1B) and OP1-induced leak currents (not shown). After a repetitive period of fluctuation, PP and OP1-induced leak currents increased progressively until the seal broke or irreversible morphological changes were induced. In the other 20% of the experiments the partial recovery was not obtained and the PP- and OP1-induced leak

currents resembled that of gramA-induced leak current (Fig. 1C) that showed a monotonous progressively increasing leak current.

3.2. Ion selectivity of PP- and OP1-induced pores

Current-voltage (I-V) relationships were constructed by measuring the inward and outward leak currents induced by the peptides from Fig. 1A. Current measurements were made at the end of each test pulse where the voltage activated Na^+ and Ca^{2+} channels are inactive ensuring that these ion channel currents are disregarded. The control curves in Fig. 2A displayed a common N-shape (polynomial function of the third order) with a E_{rev} of -80 ± 1 mV (n=10). Furthermore, an inward leak current of no greater than 0.5 nA at -100 mV and an outward leak current smaller than 0.6 nA at more positive potentials was measured. In the presence of Cs^+ as substitute for K^+ (IS_2) (data not shown) and with zero-Cl^- internal solution (IS_3) (Fig. 2C) the control curves were slightly more linear. In all cases addition of the peptides increased the linearity of the I-V relationship. Large inward and outward leak currents of approximately -3 nA (at -100 mV) and 1-2.5 nA (at +40 mV) were induced, respectively. These effects were independent of the solutions used. All peptides added to the bath caused a depolarizing shift in the E_{rev}.

In the presence of ES_1 (NaCl-based) and IS_1 (KCl-based), the addition of PP and OP1 caused a shift in E_{rev} to -7.16 ± 1.3 mV (Fig. 2A; open circles) and -7.77 ± 1.1 mV (Fig. 2B; open circles), respectively. The E_{rev} induced by PP and OP1 differed from the theoretical Nernst potential of Na^+ (+65.93 mV), K^+ (-81.99mV), Ca^{2+} (+7.4 mV) and Cl^- (-0.17 mV) calculated in accordance to these experimental conditions. Therefore, more than one ion is responsible for PP and OP1-induced leak currents. GramA was used as a positive control for a cation-selective pore [10; 11] and was tested with ES_1 (NaCl-based) and IS_1 (KCl-based). The E_{rev} shifted from -80.0 ± 0.1 mV to -25.67 ± 1.99

mV (Fig. 2D). The E_{rev} of PP and OP1 under the same conditions showed statistical significant differences to that of gramA ($p < 0.05$).

The different E_{rev} of PP and OP1-induced leak currents from the values calculated in accordance with the Nernst equation validated further experiments to determine the contributions of certain monovalent ions. Extracellular NaCl-based ES_1 was substituted with the NMDGCl-based ES_2 to determine the contribution of Na^+ to the current induced by the peptides. PP shifted the E_{rev} from -80 ± 1 mV to -12.47 ± 1.1 mV (Fig. 2A; grey circles), whereas OP1 shifted the E_{rev} to -18.96 ± 2.7 mV (Fig. 2B; grey circles), respectively. PP induced a larger shift in the E_{rev}, although it is statistically insignificant ($p < 0.05$) to that obtained by OP1. The E_{rev} obtained in the presence of the NMDGCl-base ES_2 differs statistically ($p < 0.05$) from the NaCl-based ES_1. Intracellular K^+ was substituted with Cs^+ (IS_2) to determine K^+'s contribution to PP and OP1-induced leak currents. The E_{rev} shifted from -80 ± 1 mV to -6.0 ± 1.0 mV by PP and to -3.68 ± 0.4 mV by OP1 (I-V relationship not shown). The E_{rev} of PP and OP1 in the presence of KCl-based IS_1 was not significantly different to that of the CsCl-based IS_2 ($p < 0.05$). PP shifted E_{rev} to a larger degree than OP1 when IS_1 was substituted with IS_2, although the difference is statistically insignificant ($p < 0.05$). PP was also tested in the absence of intracellular Cl^- which was substituted with aspartic acid (IS_3) (Fig. 2C). A large Cl^- concentration gradient now exists whereas in the other experiments the intra and extracellular Cl^- concentrations are similar. The E_{rev} shifted from -80 ± 0.5 mV in control to 0.4 ± 0.5 mV in the absence of intracellular Cl^-. The E_{rev} obtained in the absence of intracellular Cl^- differed with statistical significance ($p < 0.05$) from the E_{rev} of -7.16 ± 1.3 mV obtained with intracellular Cl^-.

3.3 Pore size

The estimated pores sizes of PP and OP1-induced pores in cardiac myocytes in various osmotic protectants after 50 min are shown in Fig. 3. At a concentration of 0.5 μM PP (Fig. 3; black bars) the percentage round cells ranged between 55.7 ± 9.2 % for EG and 75 ± 3.4 % for PEG 600 which differed statistically ($p < 0.05$) from the control (12.8 ± 1.6 %). The exception was PEG 1000 which had comparative damaged cells of 12.9 ± 1.6% to the control (statistically insignificant to the control). Similar results were obtained for 1 μM OP1 (Fig. 3; grey bars).

4. Discussion

The results indicate that PP and OP1 induce pores in the membrane of rat cardiac myocytes at concentrations in the micromolar range. These observations are consistent with Verdonck et al. [34] who reported pore forming activity of PP in rat dorsal root ganglion cells at a concentration of 1-2 μM and Moerman [18] who reported Ca^{2+} influxes at a concentration of 8 μM OP1 in granulocytes. There is a good correlation between concentrations used in this study and previous studies [18; 34]. The slight differences could be explained by the variations in membrane lipid composition among rat cardiac myocytes, rat dorsal root ganglion cells and human granulocytes [29]. PP is, therefore, the more toxic of the two peptides, resulting in pore-formation and hypercontracture of cardiac myocytes at lower peptide concentrations. Both peptides induced similar shifts in leak currents indicating the same type of pores.

The time course of leak current induction by PP and OP1 showed similar findings and contrasts with that of gramA. Pore formation of scorpion venom was previously investigated by Badenhorst [1] and Du Plessis [5]. They found that *O. carinatus* crude venom induced leak current with periods of fluctuations in rat cardiac myocytes. The fluctuations seen in the presence of the crude venom was

also seen in the presence of the synthetic peptide OP1 used in this study. OP1 is a peptide that has been isolated from *O. carinatus* crude venom and it may be responsible for the fluctuations of the leak current. The formation of transmembrane pores is a dynamic process that depends on a variety of factors, the electrostatic and hydrophobic interactions, the composition of the phospholipid head groups and the fatty acid chains, peptide-to-lipid ratio [14; 2]. Berchinger [2] further describes that the activity of membrane-interacting peptides as being a multi step process of accession to the membrane, bilayer association, insertion and pore-formation: water soluble ↔ surface accessible ↔ surface association ↔ bilayer inserted ↔ openings. From this approach it can be speculated that the peptide monomers are able to dislodge from the transmembrane pore and accumulate on the membrane's surface, possibly causing the fluctuations in leak current observed in Fig. 1B. A critical peptide-to-lipid concentration is also required for the formation of transmembrane pores [14], but the stability of the pore may depend on the degree of electrostatic and/or hydrophobic interactions and therefore the pore may disappear. Lee et al. [14] also stated that only alamethicin and its analogues form barrel-stave pores and that perhaps most cationic antimicrobial peptides form toroidal pores. If this is the case, PP and OP1 would have weak interactions with the phospholipid head groups, causing unstable pores and fluctuations in peptide-induced leak currents.

GramA has been shown to form cation selective pores with a permeability sequence $H^+ > NH_4^+ > Cs^+ > Rb^+ \geq K^+ > Na^+ > Li^+$ in lipid bilayers [21; 36]. GramA's E_{rev} of -25.67 ± 1.99 mV is assumed to be the E_{rev} of a monovalent cation selective pore in this experimental condition. The E_{rev} of PP and OP1-induced leak currents are different to that of gramA-induced leak current, indicating a difference in selectivity to gramA. The characterization of the pores formed by identifying the contribution of Na^+, K^+ and Cl^- towards the peptide induced leak currents was also studied. $NMDG^+$ is used to replace Na^+ and investigate its contribution to total pore induced currents [26; 28]. When

Na^+ was substituted with $NMDG^+$ the E_{rev} differed, indicating that Na^+ contributed to the PP and OP1-induced leak current. It also indicates that pores formed by PP and OP1 have a lower permeability for $NMDG^+$ than Na^+, probably due to $NMDG^+$'s larger dimensions of 1.35 x 0.55 nm [26]. The replacement of K^+ with Cs^+ indicated only slight differences in E_{rev}, which is indicative of pores having a similar permeability for K^+ and Cs^+ and that the pores do not distinguish between these two monovalent cations. The shift in E_{rev} upon substitution of intracellular Cl^- with aspartic acid indicates that PP-induced pores are permeable to Cl^-. PP-induced pores have similar selectivity for cations and anions indicating the formation of non-selective pores.

The estimation of the pore size is based on the principal of colloid osmotic shock-induced cell damage [15]. By using the morphology of the cardiac myocyte as indication of cell damage [25; 33], it may be concluded that cell damage was induced by either of the peptides when all the osmoticants up to PEG 600 (1.38 nm) were present. The cell damage was prevented when PEG 1000 (1.78 nm) was used, indicating that PEG 1000 is not able to move through the peptide-induced pores. Therefore, it was estimated that the PP and OP1-induced pores have a diameter between 1.38 nm and 1.78 nm. This estimated pore size could be the reason for the shift in E_{rev} induced by the substitution of Na^+ with $NMDG^+$, which has dimensions of 1.35 x 0.55 nm [26].

5. Conclusion

These results indicate that PP and OP1 are able to insert reversibly into the membrane of cardiac myocytes thereby inducing non-selective pores that result in fluctuating leak currents of a combination of Na^+, K^+, Ca^{2+} and Cl^-. The pores are estimated to be between 1.38 nm and 1.78 nm in diameter in cardiac myocytes membranes. In the venom gland, antimicrobial peptides play a role in the protection against microbial invasion. The formation of large, non-selective pores in cardiac myocytes (as well as nerve membranes) allows for the trafficking of ions across the membrane

leading to depolarization. This depolarizing action of such peptides could increase the potency of the venom by inducing repetitive firing, hyperexcitation and the induction of a sharp pain.

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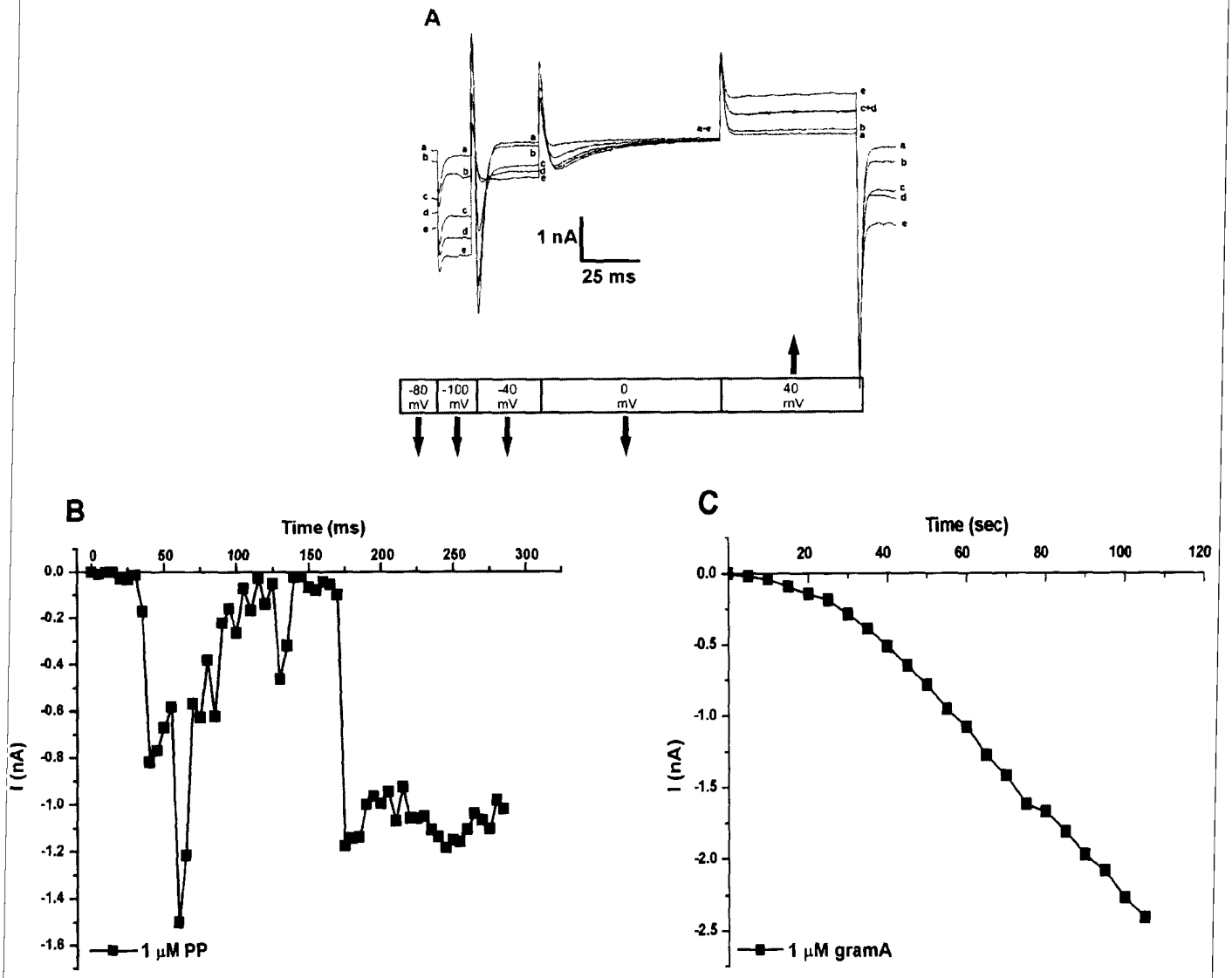

Fig. 1: (A) Pore formation by PP (1 µM) as indicated by inward (-100, -80 and -40 mV) and outward (+40 mV) currents. The control trace (a) and maximum effect (e) after 15 min is illustrated. The insert indicates the potentials at which the leak currents were recorded and the arrows show the direction in which the PP-induced current increased. PP-induced leak current at -80 mV was measured and plotted over time. Fluctuations in PP-induced leak current (B) can be observed while gramA-induced leak current (C) showed a constant increase. The time indicated in Fig. 1B and 1C is from the onset of leak current and not from the time PP and gramA were administered to the bath.

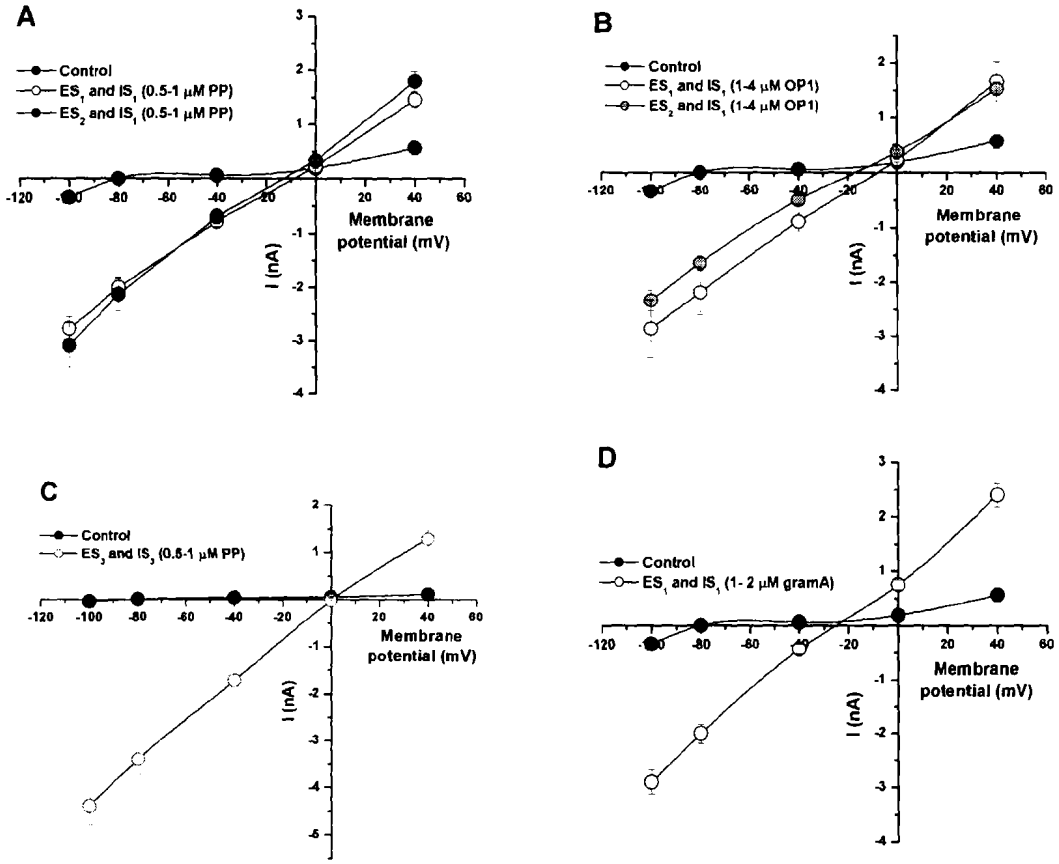

Fig. 2: Cardiac myocytes were superfused with solutions indicated in the legends and an I-V relationship of PP- (A and C; n=5), OP1- (B; n=5) and gramA-induced leak currents (D; n=5) were constructed. PP and OP1-induced leak currents were tested under various extra and intracellular solutions (see legends). GramA was only tested in presence of IS_1 and ES_1.

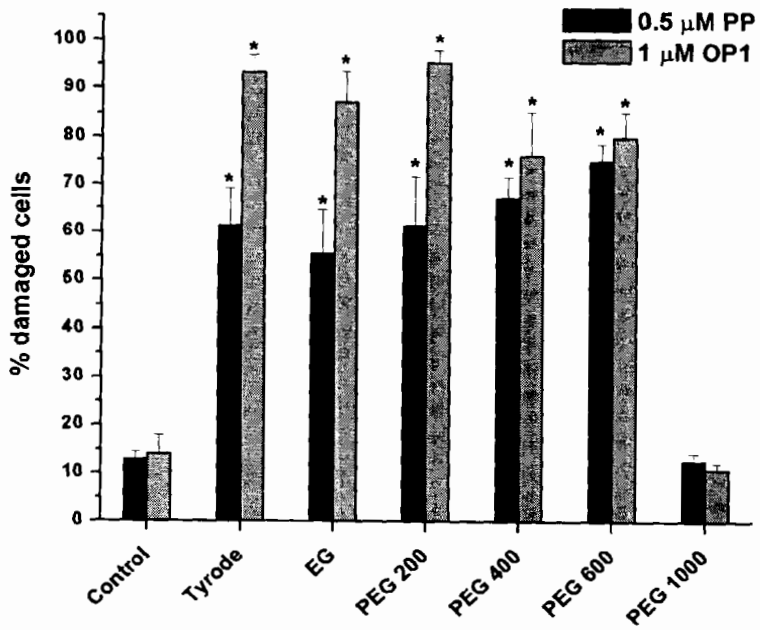

Fig. 3: Cardiac myocyte osmotic protection assay after 50 min where PP (black) and OP1 (grey) was added to the indicated solutions. Each value represents the mean ± SEM of 5-8 experiments. * $p < 0.05$ vs. control.

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Chapter 4

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Title

A confocal microscopy study of membrane potential changes induced by scorpion pore-forming toxins.

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Abstract

Scorpion venom contains ion channel selective peptides (toxins) as well as peptides that interact with the cell membrane. Parabutoporin (PP) and opistoporin 1 (OP1) are cysteine-free peptides isolated from the venom of two southern African scorpion species. At micromolar concentrations, these peptides have the potential to form transmembrane pores. The pores are dynamic in nature and allow for the trafficking of ions in and out of the cell. This ionic movement causes the membranes of cells to depolarize which was detected and quantified in a neuroblastoma cell line with the

potentiometric fluorophore TMRM and confocal microscopy. The depolarizing effect induced by the peptides was also compared to that obtained in cardiac myocytes, which showed areas of depolarization different to that obtained in the neuroblastoma cell line.

Keywords

Scorpion peptides, pore-forming, membrane potential, TMRM, confocal microscopy

Abbreviations

HEPES - 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonicaci; EGTA - ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MP - membrane potential; RMP - resting membrane potential; E_K - equilibrium potential of K^+; TMRM – tetramethylrhodamine methylester.

Introduction

Scorpion venom contains peptides that target the ion channels found in the membranes of cells (Possani et al., 2000) as well as peptides that target the membrane itself (Verdonck et al., 2000). These membrane-targeting peptides cause the formation of pores in the membrane or total disruption of it. The peptide-induced pores are permeation pathways for ions and the depolarization in neural cells function as a potential generator, triggering action potentials (Verdonck et al., 2000). PP and OP1 are two pore-forming peptides isolated from the southern African scorpion species *Parabuthus schlechteri* Purcell, 1899 (Verdonck et al., 2000) and *Opistophthalmus carinatus* Peters, 1861 (Moerman et al., 2002), respectively. It has been indicated that PP shifted the MP of human granulocytes in a depolarizing direction (Moerman, 2002).

The MP refers to the potential difference between the intra and extracellular aqueous phases of a cell and originates from the non equilibrium fluxes of ions across a membrane containing ion-selective

channels (Plášek & Sigler, 1996). During a state of rest, the MP is determined by the outflux and influx of K^+ and Na^+, respectively, as well as the contribution of the Na^+-K^+-pump (Guyton & Hall, 2000). The resting membrane potential (RMP) of neuroblastoma cell lines and cardiac myocytes vary between -48 and -20 mV (Arcangeli et al., 1995; Sacconi et al., 2005) and -82 ± 8 mV, respectively (Kohmoto et al., 1997). These ranges largely depend on the permeability properties of the membrane under question and the transmembrane ionic concentrations.

Monitoring changes in MP can be performed with a variety of techniques. Electrophysiological techniques (current-clamping) can be used to measure MP and changes brought about by various factors, provided the cells are large enough for the formation of electrode-membrane junctions. Even with large cells, the accuracy of this technique can be affected by ionic leak currents at the electrode-membrane junction. Other limitations have also been quoted in the literature (Ehrenberg et al., 1988; Plášek & Sigler, 1996). Potentiometric fluorophores are advantageous and an accurate way of investigating changes in MP. Tetramethylrhodamine methylester (TMRM) is a fluorophore that enables MP measurement without the disadvantages of other potentiometric fluorophores, namely, photobleaching and toxicity towards the cells (LeMasters et al., 1999). The potential difference across the membrane drives an uneven distribution of TMRM between the intra and extracellular mediums and this fluorophore will fluoresce intensely at more negative MP due to this fluorophore's cationic nature, for example, at negative MP of ~-90 mV the fluorophore will fluoresce intensely and depolarization of the membrane will show less TMRM fluorescence. Often potentiometric fluorophores deviate significantly from the Nernst equation due to their binding to the plasma and organelle membranes and the tendency of the compounds to form aggregates when their concentrations reach a threshold. TMRM shows none of these characteristics (Loew et al., 2002) and therefore the fluorophore distribution and intensity equilibrates in accordance to the Nernst equation:

$$MP = -60\log\left(\frac{[\text{fluorophore}]_{intra}}{[\text{fluorophore}]_{extra}}\right) = -60\log\frac{\text{fluorescence}_{intra}}{\text{fluorescence}_{extra}}\,mV$$

In the present study, TMRM and digital imaging (confocal microscopy) were used to attempt to quantify MP changes induced by PP and OP1 in neural (SH-SY5Y neuroblastoma cell line) and muscle cells (rat cardiac myocytes).

Material and methods

Peptides and chemicals

PP (Swiss-Prot Accession No. P83312) and OP1 (Swiss-Prot Accession No. P83313) were chemically synthesized by Ansynth Service BV (The Netherlands) as described earlier (Moerman et al., 2002). Valinomycin was purchased from Sigma (St. Louis, MO, USA). TMRM was purchased from Molecular Probes (California, CA, USA).

Solutions

Loading solution (LS) (mM): 0.05 TMRM, 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 11.6 HEPES, 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. Depolarizing recording solution 1 (DRS_1) (mM): 0.01 TMRM, 137 NaCl, 5.4 KCl, 2 $MgCl_2$, 11.6 HEPES, 5 EGTA, 10 glucose, pH 7.4 with NaOH. Depolarizing recording solution (DRS_2) (mM): 0.01 TMRM, 5.4 NaCl, 140 KCl, 0.5 $MgCl_2$, 11.6 HEPES, 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. Recording solution 1 (RS_1) (mM): 0.01 TMRM, 137 NaCl, 5.4 KCl, 2 $MgCl_2$, 11.6 HEPES, 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH. Recording solution 2 (RS_2) (mM): 0.01 TMRM, 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 11.6 HEPES, 1.8 $CaCl_2$, 10 glucose, pH 7.4 with NaOH.

Enzymatic isolation of rat ventricular cardiac myocytes and TMRM loading

Ventricular cardiac myocytes were isolated from Sprague-Dawley rats (~200 g) using the enzymatic dispersion method developed by Mitra and Morad (1985) and revised by Tytgat (1994). 24 mg collagenase (type II, Sigma, St. Louis, MO, USA) and 4.8 mg protease (type XIV, Sigma, St. Louis, MO, USA) were used. Cardiac myocytes were allowed to adhere for 1 hour at room temperature to poly-L-lysine and laminin laminated sterile 31 mm cover slips. The cells were then allowed to load with 0.5 μM TMRM in loading solution for 20 min (Loew et al., 1998) in a sterile 35 mm petri dish after which they were placed in the cell chamber. Depolarizing recording solution 1 (DRS_1) or recording solution 1 (RS_1) were used for MP recordings.

Preparation of SH-SY5Y neuroblastoma cell line and TMRM loading

SH-SY5Y neuroblastoma cells were seeded in Dulbecco's modified Eagle's medium (DMEM-F12) and kept in culture flasks in a 5% CO_2 incubator at 37°C. Cells were trypsinized and seeded again for 5 hours onto sterile 31 mm cover slips in sterile 35 mm petri dishes and allowed to grow further in DMEM-F12 in a 5% CO_2 incubator at 37°C. The cells were loaded with 0.5 μM TMRM for 20 min (Loew et al., 1998) in LS. Depolarizing recording solution 2 (DRS_2) or recording solution 2 (RS_2) were used for MP recordings of the neuroblastoma cells.

Confocal microscopy

Digital images of MP changes were obtained with a model PCM 2000 confocal microscope as proposed by Ehrenberg et al., 1988 and Loew et al., 1998. The PCM 2000 was connected to a Nikon (TE300) inverted microscope equipped with a 60x/1.40 Apo Planar oil objective. The system's helium-neon ion laser was used to excite TMRM at 515 nm and emission waves were obtained at 565 nm. A 10% neutral density filter was used to distinguish the plasma MP from the mitochondrial MP. A pinhole size of 1/4 was used to minimize photobleaching. The gain was set to ensure the

brightest fluorescence (mitochondria MP) was < 250 intensity units. This enabled photometric measurements to be possible. A scan rate of 1 frame/s was chosen and a total of 8 frames were averaged.

Quantification of results

Quantification of the results obtained from the neuroblastoma cell line were performed according to Loew et al., 1998 and LeMasters et al., 1999. Briefly, cells were depolarized with DRS_2 in the presence of 1 μM valinomycin to bring the MP close to that of K^+'s equilibrium potential (E_K). At E_K the extracellular over intracellular TMRM fluorescence ratio (F_{extra}/F_{intra}) was calculated. This corrected for any non-potentiometric binding of the fluorophore. Intra and extracellular intensities before and after PP or OP1 exposure were obtained and the Nernst equation used to calculate MP (Loew et al., 1998).

Changes in MP of cardiac myocytes were performed according to Moerman (2002). As DRS_2 caused morphological changes (long to spherical shape) in the cardiac myocytes, DRS_1 with 4 different extracellular K^+ concentrations had to be used for the calculation of a calibration curve. The sum of the Na^+ and K^+ concentration was kept the same in all 4 of the extracellular K^+ concentrations.

Statistical analysis

Digital images were analysed with the EZ2000 software (Nikon, Japan). Results were expressed as the mean ± SEM. The significance of the differences was expressed using Student's paired and unpaired t-test in Origin, version 5.0 (Microcal Software Inc., © 1991-1997). In all analysis values of $p < 0.05$ were considered statistically significant.

Results

High extracellular K^+ depolarized neuroblastoma and cardiac myocytes

The K^+-specific ionophore valinomycin has been extensively used in cell biology to clamp the plasma membrane potential to the K^+ equilibrium potential (Farkas et al., 1989). In the case of the neuroblastoma cell line DRS_2 (with 1 μM valinomycin) (Fig. 1B) caused a F_{extra}/F_{intra} ratio of 0.2 ± 0.01 (n = 7). According to the Nernst equation these conditions should hold the MP to 0 mV, considering an intracellular K^+ concentration of 140 mM (Guyton & Hall, 2000). The MP obtained in DRS_2 (with 1 μM valinomycin) after 30 min was -0.85 ± 1.5 mV (n = 7) (data not shown).

The four extracellular K^+ concentrations (DRS_1 with 1 μM valinomycin), induced a loss of TMRM fluorescence in some areas on the cardiac myocyte. The membrane did not lose its TMRM fluorescence uniformly, therefore, 20-30 mins were allowed to pass until a large portion or the entire cell lost its fluorescence before intracellular intensity readings were taken (Fig. 1D). The percentage fluorescence decrease readings were plotted against the corresponding logarithmic function of the extracellular K^+ concentration. A near perfect straight-line fitting was made through the four points (r = 0.98) (data not shown). These values were then correlated to the Nernstian values and Fig. 1E indicates that a good correlation occurs between the theoretically calculated MP values (Nernst equation) and the intensity readings obtained. Fig. 1F was constructed to enable the calculation of the MP for a decrease in TMRM fluorescence.

Effect of PP and OP1 on membrane potential

PP and OP1 have been shown to allow the flow of ions in and out of the rat dorsal root ganglion cells (Verdonck et al., 2000), human granulocytes (Moerman, 2002) and cardiac myocytes (Elgar et al., unpublished). Therefore, decreased TMRM fluorescence in neuroblastoma cells and cardiac

myocytes were expected. The intensity readings were taken as shown in Fig. 2 (according to Loew et al., 1998) and substituted into the Nernst equation as explained previously. The RMP of -38.3 ± 1.9 mV (n = 7) was calculated for the neuroblastoma cells. After 20-30 min of peptide exposure time, 0.5 μM PP and 1-1.5 μM OP1 caused a decrease in TMRM fluorescence. PP (Fig.2) and OP1 (data not shown) depolarized the MP to -11.9 ± 3.9 mV (n = 7) and -9.4 ± 1.9 mV (n = 7), respectively. The time to effect was dependant on the concentration peptide administered to the cells and all changes in MP were statistically significant ($p < 0.05$) from the control.

In cardiac myocytes PP (Fig. 3) and OP1 (data not shown) caused a reduction in TMRM fluorescence at a concentration of 0.5-1 μM and 1.5-2.5 μM, respectively. PP caused effects after 10-25 min whereas OP1 after 20-35 min. It was also indicated that the time to effect was concentration dependant. The RMP of the cardiac myocytes was –83.8 ± 8 mV (n=5) (Fig.3A). PP and OP1 shifted the MP to -39.7 ± 8.4 mV (n = 5) and -32.6 ± 5.2 mV (n = 5), respectively. Changes in MP were statistically significant ($p < 0.05$) from the control.

Discussion

The formation of peptide-induced pores has been proven by a number of researchers with a number of cell types with a variety of techniques (Béven et al., 1998; Ibrahim et al., 2001; Moerman, 2002). The fact that PP and OP1 interact with membranes of excitable cells, causing the formation of pores and trafficking of ions leads to the discovery that these peptides caused changes in the MP of human granulocytes (Moerman, 2002). The current study took a step further in quantifying the depolarizing effect of the peptides in a neuroblastoma cell line and cardiac myocytes.

The MP of -0.85 ±1.5 mV calculated for the neuroblastoma cells in a high extracellular K^+ concentration is within a narrow range of the expected E_k of 0 mV and validates the method used in calculating plasma MP in this cell type. The same cannot be said for the cardiac myocytes as the cells morphologically changed from long to spherical cells in such high extracellular K^+ concentration. Therefore, an alternative method, as described in the Material and Methods was used.

Previous studies have shown that the pores induced by PP and OP1 allowed the flow of ions across the membrane of cells (Verdonck et al., 2000; Du Plessis, 1999) and this being the reason for the change in MP. PP caused a change in the MP at lower concentrations, indicating that PP is more toxic towards the neuroblastoma cell line and cardiac myocytes than OP1. The RMP of both cell types obtained in the study corresponds well with that of the literature, adding more credability to the results obtained via both methods used (Arcangeli et al., 1995; Kohmoto et al., 1997; Sacconi et al., 2005). The neuroblastoma cells (Fig. 2) and cardiac myocytes (Fig. 3) were depolarized from the negative RMP (indicated by bright TMRM fluorescence) to a less negative MP (indicated by reduced TMRM fluorescence) (Plášek & Sigler, 1996). The peptides either caused a net influx of positive ions or a net outflux of negative ions causing the depolarizing effect in both cell types.

The formation of pores in the membrane and/or disruption of the membrane could enhance the actions of other venom components (Corzo et al., 2001). It is speculated that the peptides, by depolarizing neural cells, contribute to the induction of pain associated with scorpion stings (Verdonck et al., 2000). The fact that these peptides are able to interact with and cause depolarization of the membranes of muscle cells, allows the speculation that they could contribute to a hypercontracted state of the muscle, that could lead to paralysis and convulsions of smaller prey. These peptides could, therefore, form part of the scorpion's offensive armour used to capture its prey. In addition to eukaryotic cell membranes, these peptides are active against bacterial

membranes as well. Many scorpion species have been known to dig burrows in bacteria-rich soil in which they spend many hours of the day (Leeming, 2003). These peptides could, therefore, serve as a defense mechanism against bacterial infection of the scorpion's venom glands.

In conclusion, PP depolarized the SH-SY5Y neuroblastoma cell line and rat cardiac myocytes at lower concentrations than OP1. It can also be concluded that digital imaging of TMRM allowed for reliable quantifying of MP changes.

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Figures and Captions

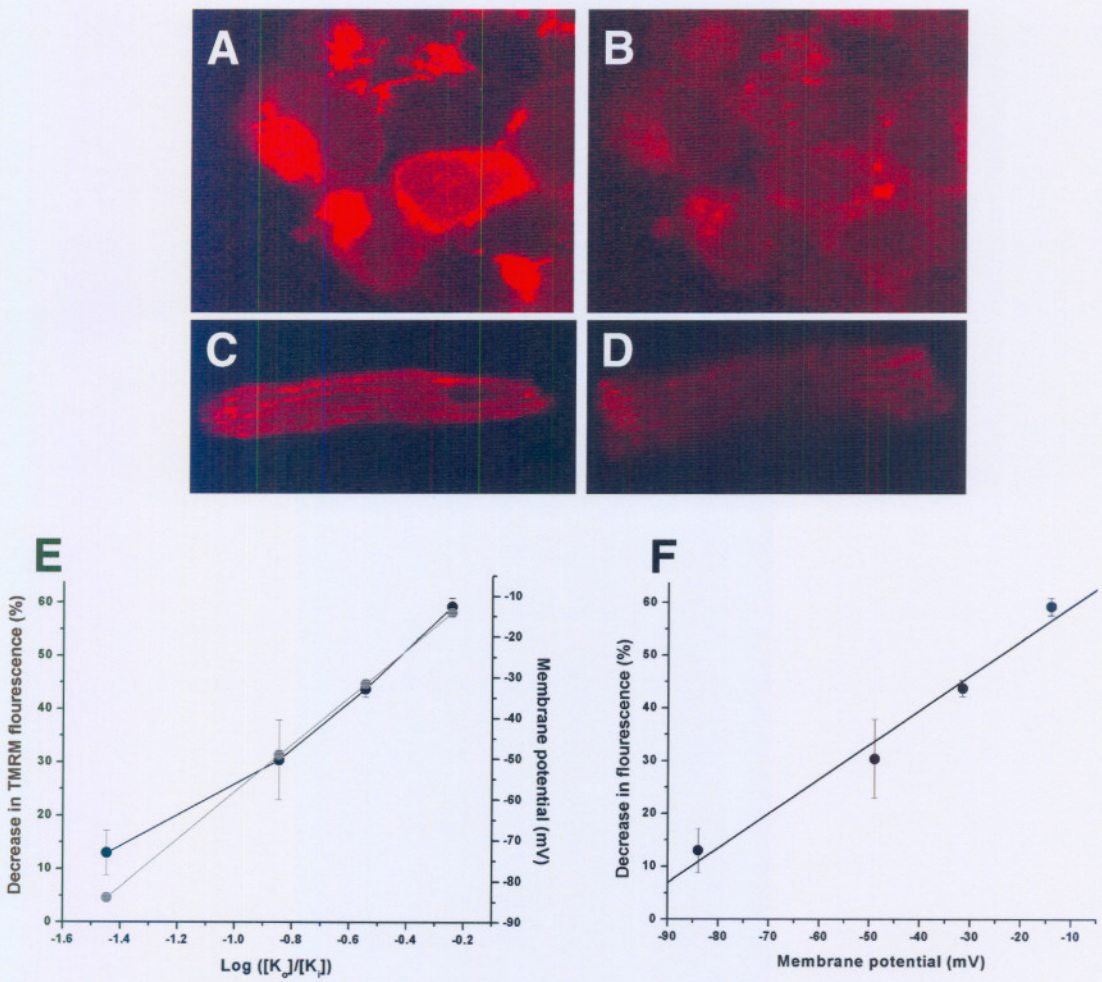


Fig. 1: Effect of high extracellular K^+ (with 1 µM valinomycin) on neuroblastoma cells and cardiac myocytes. Neuroblastoma cells (A) and cardiac myocytes (C) shown with a bright TMRM fluorescence (control/RMP) and then in the presence of $DRS_{1/2}$ with 1 µM valinomycin (B and D, respectively). (E) The correlation of this decreased TMRM fluorescence and MP was plotted. The grey and black line indicates the measured change in TMRM fluorescence (left axis) and the MP expected from the Nernst equation (right axis), respectively. (F) Correlation of % decrease in TMRM fluorescence and MP in cardiac myocytes (see Material and Methods for details).

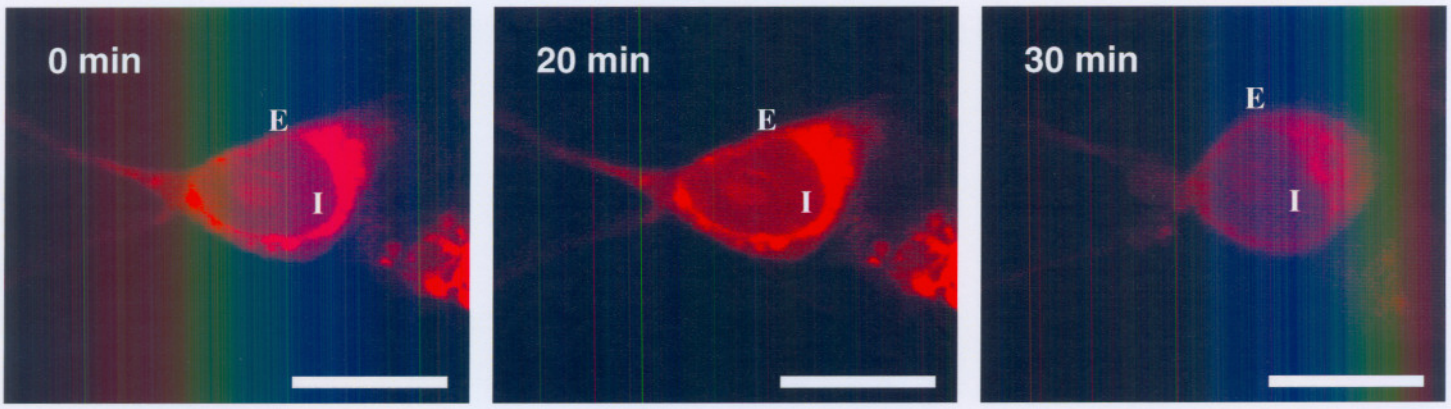


Fig. 2: Confocal microscopy images of TMRM labelled SH-SY5Y neuroblastoma cells. 0.5 μM PP was administered at time 0 min. After 20 min of exposure to the peptide the MP had not altered from the RMP. After 30 min of exposure the fluorescence decreased. The extracellular and intracellular TMRM intensities were measured at position 'E' and 'I', respectively. (Bar indicates 10 μm.)

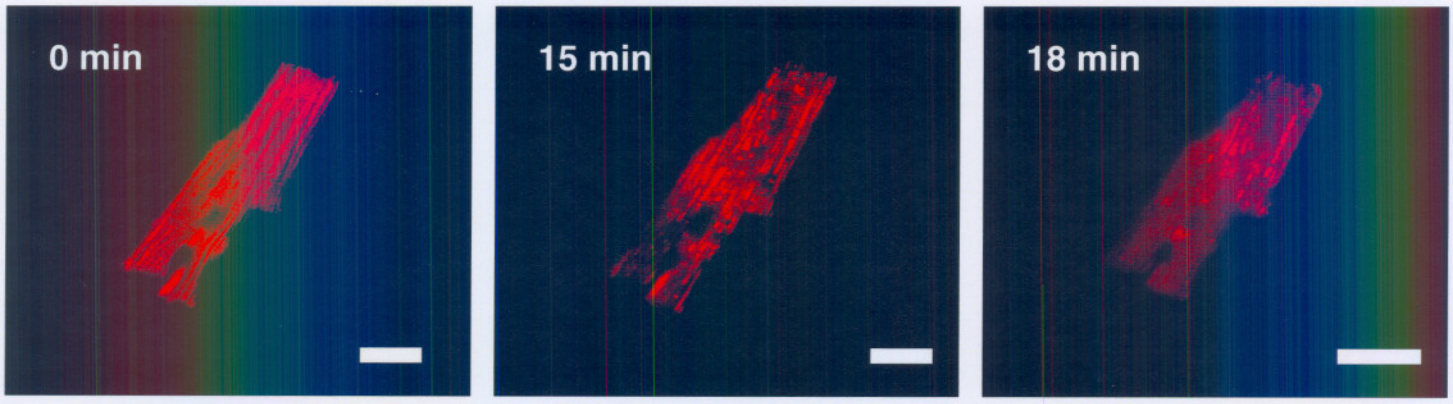


Fig. 3: Confocal microscopy images of TMRM marked cardiac myocytes. 0.5 μM PP caused the fluorescence of TMRM to decrease in areas over the cell to result in a uniform distribution after ~18 min. (Bar indicates 15 μm.)

Appendix

Note: IU = Intensity Units

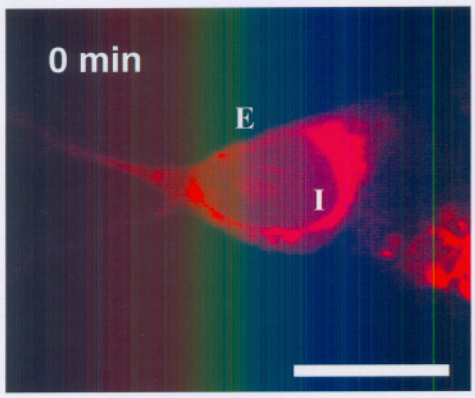
Calibration ratio for neuroblastoma cell line (140 mM K^+ solution with 1 µM valinomycin)

Extracellular fluorescent intensity / Intracellular fluorescent intensity

F_{extra}/F_{intra} = 5 IU / 25 IU

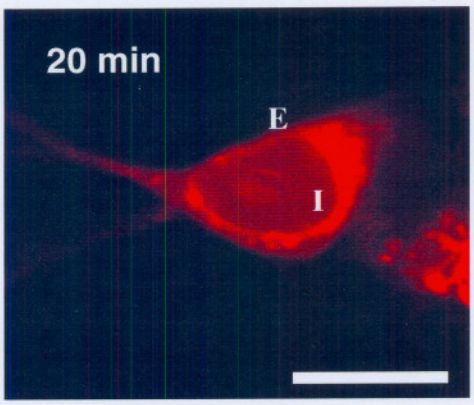
= 0.2 ± 0.01

Calculation of MP of neuroblastoma cells



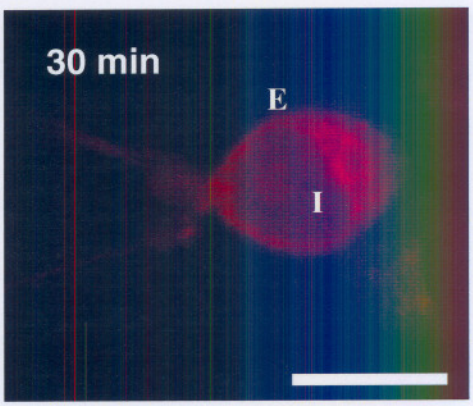
E = 5 IU

I = 115 IU



E = 5 IU

I = 115 IU



E = 5 IU

I = 35 IU

$$MP = -60\log\left(\frac{[\text{fluorophore}]_{intra}}{[\text{fluorophore}]_{extra}}\right) = -60\log\frac{\text{fluorescence}_{intra}}{\text{fluorescence}_{extra}}\,\text{mV}$$

Resting membrane potential (time 0 min)

$$MP = -60\log\frac{(115*0.2)}{5} = -39.8\text{mV}$$

Membrane potential after 30 min peptide exposure

$$MP = -60\log\frac{(35*0.2)}{5} = -8.8\text{mV}$$

Chapter 5: Conclusions and Recommendations

Antimicrobial peptides have been investigated for many years. These peptides have been found in all types of animals and humans and play an important role in the fight against infection and disease. Only since the year 2000 have these peptides been isolated from the venom of scorpions. The first peptides isolated and characterized were parabutoporin from the South African specie *P. schlechteri* and hadrurin from the Mexican specie *H. aztecus*. To date, 14 linear α-helical antimicrobial peptides and 2 cationic, cysteine-rich antimicrobial peptides have been isolated from the venom of scorpions.

These peptides target the membranes of bacterial, fungal and mammalian cells. Peptide aggregation on membranes allows for the formation of transmembrane pores, a proposed mechanism of cell death by causing fatal depolarization or the leakage of critical cell content to the extracellular environment.

Many peptide-induced pores have been reported to be permeable towards either cations, anions or both, indicating non-selectivity. When primary cell cultures are used for these types of investigations, the shift in the reversal potential (E_{rev}) is used as indication of ion selectivity of peptide-induced pores. This study used the whole-cell patch clamp technique as a means to investigate the ion selectivity of the PP and OP1-induced pores in rat cardiac myocytes. Pore formation of PP and OP1 was observed at submicromolar and micromolar concentrations, respectively. An interesting observation of fluctuating PP and OP1-induced leak currents was observed in contrast to the monotonous progressively increased leak current of gramicidin A (gramA). GramA was also used as a positive control of a monovalent cation selective pore. Under identical conditions, the E_{rev}'s of PP and OP1 differed significantly from that of gramA, indicating that PP and OP1 were selective towards other ions as well. In the manipulation of extracellular Na^+ and intracellular K^+ and Cl^-, it was made possible to conclude that the selectivity of PP- and OP1-induced pores were non-selective. Therefore, the first part the hypothesis stating that PP and OP1 induce non-selective pores in rat cardiac myocytes is accepted.

The estimate size of the pores was determined by an osmotic protection assay. The results showed the estimated pore size of PP and OP1 to be between 1.38 nm and 1.78 nm. The size of the pores would allow for easy flow of ions in and out of the cell. It could also explain the shift in E_{rev} in the presence of extracellular $NMDG^+$, as the size of this molecule is within narrow range of the size of the pores.

The membrane potential (MP) effect of PP and OP1 was investigated on a SH-SY5Y neuroblastoma cell line and primary rat cardiac myocytes. The neuroblastoma cells proved to be a reliable cell type for the quantification of MP changes as the fluorescence decreased uniformly throughout the cell. Valinomycin in an extracellular solution containing 140 mM K^+ was used to calibrate the response of TMRM to the depolarization of PP and OP1. According to the Nernst equation (accepting the intracellular K^+ concentration of the cells is 140 mM) the MP should be 0 mV, which corresponds well with the reading of -0.85 ± 1.5 mV recorded. This showed that the method followed was correct. PP and OP1 depolarized the cells from the resting MP (RMP) of -38.3 ± 1.9 mV to -11.9 ± 3.9 mV and -9.4 ± 1.9 mV, respectively, at micromolar concentrations. The quantification of MP changes in the cardiac myocytes proved to be more difficult as the fluorescent intensity was lost in areas of the cells and not uniformly throughout the cell. The calibration of the TMRM also proved to be a lengthy process where four different extracellular K^+ concentrations had to be used for the construction of a plot indicating the relationship of decrease in TMRM fluorescence as a function of the extracellular K^+ concentrations. Time had to pass until the entire cell or a large portion of the cell lost its fluorescence to enable the reading of the fluorescence. The peptides depolarized the cardiac myocytes from the RMP of -83.8 ± 8 mV to -39.7 ± 8.4 mV (0.5-1 µM PP) and -32.6 ± 5.2 mV (1.5-2.5 µM OP1). The MP of the neuroblastoma cells are within a narrow range to the E_{rev}'s recorded with the cardiac myocytes. The second part of the hypothesis that PP and OP1 induce depolarization of the target cell is accepted.

For future studies, it is recommended that the non-selectivity of PP and OP1-induced pores be confirmed with lipid bilayers and single channel recordings. Lipid bilayers are biological membranes that separate artificial compartments. This is advantageous in that the extra and intracellular solutions may be easily manipulated without the problem of causing unfavourable conditions for cells i.e. cardiac myocytes. Single channel recordings could give insight to whether the pores possess any unique opening and closing kinetics. A further recommendation is to perform similar pore size experimentation at different concentrations of PP and OP1. This will determine whether the size of the pores increases with increased peptide concentration, indicating a dynamic or static pore size. Current-clamp may also be used to verify the changes in MP obtained with the potentiometric fluorophore TMRM.