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Toxicon 40 (2002) 111–124

Review

TOXICON

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Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria)

Gregor Anderluh, Peter Maček*

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111,1000 Ljubljana, Slovenia

Received 20 March 2001; accepted 15 July 2001

Abstract

More than 32 species of sea anemones have been reported to produce lethal cytolytic peptides and proteins. Based on their primary structure and functional properties, cytolysins have been classified into four polypeptide groups. Group I consists of 5–8 kDa peptides, represented by those from the sea anemones *Tealia felina* and *Radianthus macrodactylus*. These peptides form pores in phosphatidylcholine containing membranes. The most numerous is group II comprising 20 kDa basic proteins, actinoporins, isolated from several genera of the fam. Actiniidae and Stichodactylidae. Equinatoxins, sticholysins, and magnificalysins from *Actinia equina*, *Stichodactyla helianthus*, and *Heteractis magnifica*, respectively, have been studied mostly. They associate typically with sphingomyelin containing membranes and create cation-selective pores. The crystal structure of equinatoxin II has been determined at 1.9 Å resolution. Lethal 30–40 kDa cytolytic phospholipases A_2 from *Aiptasia pallida* (fam. Aiptasiidae) and a similar cytolysin, which is devoid of enzymatic activity, from *Urticina piscivora*, form group III. A thiol-activated cytolysin, metridiolysin, with a mass of 80 kDa from *Metridium senile* (fam. Metridiidae) is a single representative of the fourth family. Its activity is inhibited by cholesterol or phosphatides. Biological, structure–function, and pharmacological characteristics of these cytolysins are reviewed.

Keywords: Cytolysin; Hemolysin; Pore-forming toxin; Actinoporin; Sea anemone; Actiniaria; Review

1. Introduction

Coelenterates (Cnidaria) produce a variety of peptides and proteins that act as neurotoxins or cytolysins. For hydrozoan, scyphozoan and anthozoan species, there is evidence that toxins are delivered by nematocysts (cnidocysts), stinging organelles characteristic for cnidarians (Hessinger and Lenhoff, 1988). Despite the belief that all cnidarians are venomous or poisonous, biochemical and pharmacological studies have focussed on only a few groups. Of considerable interest have been cytolytic toxins from several jellyfish species and the siphonophore, the Portuguese man-of-war (*Physalia physalis*), that threatens human health (Calton and Burnett, 1988). Being highly unstable, jellyfish toxins are biochemically less well characterized than those of anthozoans. However, box jellyfish (g. *Carybdea*) cytolysins have been cloned and sequenced (Nagai et al., 2000a,b). Unlike pelagic cnidarians, sedentary sea anemones have proved less harmful for humans, due to a lower incidence of stinging and sting severity. Nevertheless, blockers of sodium and potassium channels (Norton, 1991, 1998), and cytolysins lethal to vertebrates and crustaceans (Kem, 1988; Bernheimer, 1990; Turk, 1991; Maček, 1992) from a number of sea anemones (Actiniaria) have been extensively characterized. In addition, a novel type of 28 kDa cardiostimulatory protein devoid of cytolytic activity and containing cysteine residues was recently isolated. The N-terminal sequences of two such proteins from *Urticina piscivora* proved them to be different from the large family of 20 kDa cytolysins (Cline et al., 1996).

Here, we review the progress made in research on sea anemone cytolysins in the last decade. It is characteristic of that period that a number of novel toxins have been purified and characterized. There is emerging evidence that sea anemones produce at least four groups of cytolytic polypeptides. Based on their molecular weight, they can be classified as: (a) 5–8 kDa peptides with antihistamine activity, (b) ~20 kDa pore-forming proteins inhibited by

* Corresponding author. Tel.: +386-1-4233388; fax: +386-1-2573390.

E-mail address: peter.macek@uni-lj.si (P. Maček).

0041-0101/01/$ - see front matter
PII: S0041-0101(01)00191-X

Table 1
Groups of sea anemone cytolysins and their representative characteristics

Group	M.w.(kDa)	pI	Lethality (mg/kg)	Inhibitory lipid(s)	Example	Activity
I	5–8, SDS–PAGE, Amino acid analysis	> 9	> 10.0[a]	Phosphatidylcholin[e]	RmI, RmII lysin	Pore formation; Antihistamine
II	20, SDS–PAGE, Sequencing	> 9.5	0.035[b]	Sphingomyelin	Equinatoxins, Sticholysins, Magnificalysins	Pore formation
III	~30–45, SDS–PAGE	> 8.8	0.04[c] 10 μg/ml[d]	? Sphingomyelin	α, β PLA_2 (*A. pallida*), UpI (*U. piscivora*)	PLA_2-activity; Pore formation
IV	80, Gel permeation chromatography	5.0	0.32[e]	Cholesterol, Phosphatides	Metridiolysin	Pore formation

[a] Mice *i.p.* LD_{100} for RmI lysin from *Radianthus macrodactylus* (Zykova et al., 1998).
[b] Mice *i.v.* LD_{50} for EqtII from *Actinia equina* (Maček and Lebez, 1988).
[c] Crab LD_{100} for fraction III from *Aiptasia pallida* (Hessinger and Lenhoff, 1976).
[d] Fish freshwater LD_{100} for UpI from *Urticina piscivora* (Cline et al., 1995a).
[e] Mice *i.v.* LD_{50} for Metridiolysin from *Metridium senile* (Bernheimer and Avigad, 1978).
Molecular weight (m.w.) determination with SDS–PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis, amino acid analysis, sequencing and gel permeation chromatography.

sphingomyelin, (c) ~30–40 kDa cytolysins with or without phospholipase A_2-activity, and (d) a putative group of proteins represented at present solely by an 80 kDa *Metridium senile* cytolysin. Group representatives and their typical characteristics are shown in Table 1.

A remarkable breakthrough in structural and functional characterization of the 20 kDa pore-forming cytolysins has been made in terms of both their cloning and cDNA sequencing (Anderluh et al., 1995; 1996; 1999; Pungerčar et al., 1997; Wang et al., 2000; de los Rios et al., 2000) and the structural characterization of equinatoxin II from *Actinia equina* (Athanasiadis et al., 2001). Protein engineering was employed in structure–function studies. Cytolysins also serve as model proteins to study protein-lipid membrane interactions and have been examined for their use in eradicating tumour cells and parasites. We also point to the biological roles that cytolysins may have in sea anemones or other organisms.

2. Biological aspects of sea anemone cytolysins

In the last three decades, at least 32 species of sea anemones have been reported to contain one or more cytolytic peptides or proteins (Table 2). It has become evident that one species can produce a number of 20 kDa isotoxins. For example, in *Actinia equina* at least five isoforms of equinatoxin have been distinguished by protein and cDNA analysis (Anderluh et al., 1999). Moreover, *Radianthus macrodactylus* has been reported to contain both 5–8 and 20 kDa cytolysins (Zykova et al., 1998; Monastyrnaya et al., 1999). It is also evident that production of a cytolysin in a sea anemone does not exclude coexistence of sodium and potassium ion channel neurotoxins (Kem, 1988; Lin et al., 1996; Anderluh et al., 2000). In contrast to the situation with pelagic cnidarians, the exact origin of both neurotoxins and cytolysins of sea anemones remains unanswered despite the common belief that they are stored and delivered by nematocysts. So far, only very few toxins have been proven to be located in nematocysts (Calton and Burnett, 1988; Lotan et al., 1996; Grotendorst and Hessinger, 1999). Cytolytic toxins may also be secreted by intact contracting sea anemones on mechanical stimulation (Maček and Lebez, 1988; Senčič and Maček, 1990), however it has not been clarified whether they are released from nematocysts or from some other stores. For intact *Phymactis clematis*, a 20 kDa hemolysin, named coelenterolysin, has been shown to be present in the gastrocoelic liquid (Meinardi et al., 1994). It was suggested to be different from a hemolysin found in tentacle nematocysts and in the sea anemone homogenate. Moreover, Klug et al. (1988) showed that discharge of tentacle nematocysts of *A. equina*, a well-known producer of cytolysins, directly into a red blood cell suspension did not induce hemolysis, in contrast to observations for some other nematocysts of hydrozoan, and jellyfish origin. Interestingly, the severely stinging sea anemone *Anemonia sulcata* (Maretić and Russell, 1983) belonging to the family Actiniidae is devoid of any cytolytic activity (Maček, unpublished observation). Taken together these observations indicate that the storage and delivery of sea anemone toxins has yet to be clarified.

In natural habitats, sea anemones may prey on small crustaceans and fishes. On the other hand, certain species of fishes and crustaceans are known to live in symbiosis with sea anemones (Shick, 1991). Toxicological studies of purified toxins or live sea anemones, using crustaceans and fishes revealed that, at a concentration in sea water as low as 0.5 μg/ml, 20 kDa cytolysins damaged gills and killed non-symbiotic fishes. Yet, symbionts and habituated organisms acquired resistance when exposed to sea

Table 2
Survey on sea anemone species reported to produce cytolytic peptides and proteins. Data are collected from reviews of Bernheimer (1990); Turk (1991); Maček (1992), and from references in table. Classification and synonyms in parentheses are according to Shick (1991)

Family species	Toxin group	Reference
Tribe Mesomyaria		
Aiptasiidae		
Aiptasia pallida	III	(Grotendorst and Hessinger, 1999)
Metridiidae		
Metridium senile	IV	(Bernheimer and Avigad, 1978)
Tribe Endomyaria		
Actiniidae		
Actinia cari	II	
Actinia equina	II	
Actinia tenebrosa	II	
Anthopleura japonica	II	
Anthopleura xanthogrammica	II	
Anthopleura michaelseni	II	
Anthopleura fuscoviridis	II	
Bolocera tuediae	n.d.	(Calton et al., 1978)
Bunodactis marplatensis	n.d.	(Meinardi et al., 1994)
Bunodactis regnaudi	n.d.	
Bunodosoma caissarum	II	(Malpezzi and Freitas, 1991)
Bunodosoma capensis	II	
Condylactis aurantiaca	I	
Entacmea quadricolor[a]	II	(Samejima et al., 2000)
Epiactis japonica	II	
Epiactis prolifera	II	
Gyrostoma helianthus	II	
Phymactis clematis	II	(Meinardi et al., 1994)
Pseudactinia varia	II	
Pseudactinia flagellifera	II	
Tealia (Urticina) felina	I	
Tealia (Urticina) lofotensis	n.d.	
Urticina piscivora	III	(Cline et al., 1995b)
Stichodactylidae		
Heteractis magnifica	II	(Khoo et al., 1993)
Radianthus (*Heteractis*) *koseirensis*	II	
Radianthus (Heteractis) macrodactylus	I, II	(Zykova et al., 1998; Monastyrnaya et al., 1999)
Stichodactyla (*Stoichactis*) *helianthus*	II	
Stichodactyla (*Stoichactis*) *kenti*	II	
Stichodactyla mertensii	II	(Samejima et al., 2000)

[a] Syn. *Parasicyonis actinostoloides*; n.d., not determined.

anemone tentacles (Mebs, 1994). A similar situation was found with crustaceans. The chitin-coated gills of crustaceans were found to be resistant up to a cytolysin concentration of 100 μg/ml. However, toxins were lethal if injected into haemocoel (Giese et al., 1996).

With respect to the observed resistance of sea anemones to their own cytolysins (Senčič and Maček, 1990) and their intraspecies aggression behaviour such as acrorhagic responses (Shick, 1991; Bigger, 1988), there is an interesting report by Meinardi et al. (1995). These authors found that in cell membranes of sea anemones sphingomyelin is replaced by its phosphono analogue. They demonstrated that, in contrast to sphingomyelin, this analogue does not act as an acceptor for 20 kDa hemolysins and this could be the basis of self-resistance.

3. Biochemical and functional characteristics of cytolysins

3.1. Group I

Till now, 5–8 kDa cytolytic peptides have been found in *Tealia felina* (Elliott et al., 1986), *R. macrodactylus* (Zykova et al., 1998), and *Condylactis aurantiaca* (Sedmak, B. Doctoral Thesis, Ljubljana, 1993). *R. macrodactylus* 5.1 kDa RmI and 6.1 kDa RmII cytolysins have been

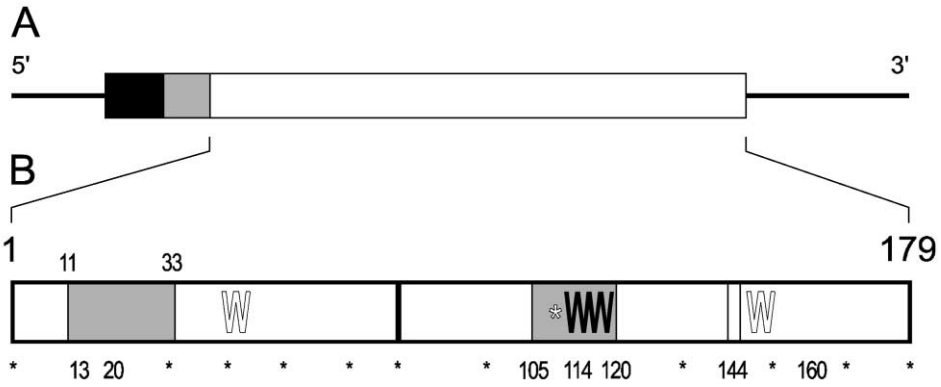


Fig. 1. Structural and functional organisation of equinatoxin II from *Actinia equina*. A. Structural organisation of EqtII cDNA (Anderluh et al., 1996). EqtII is encoded as a preproprotein. The N-terminal part contains a typical signal peptide of 19 amino acids (black) and propeptide of 16 amino acids (gray) (Anderluh et al., 2000b). The mature EqtII is a protein of 179 amino acids (white). B. Functional organisation of EqtII based on topological studies (Anderluh et al., 1998, 1999, 2000a; Malovrh et al., 2000). Regions interacting with the membrane are presented in gray. The RGD-motif is represented by a white rectangle. The position of the predicted N-terminal amphiphilic α-helix (Belmonte et al., 1994) is shown above the cartoon. Cysteine mutants that interact with the membrane in the membrane bound form are numbered below. An asterisk depicts the positions of other mutants (Anderluh et al., 1998, 1999). Tryptophan residues shown to interact with the membrane are presented in black; those that remain exposed to the solution are presented in white. A hollow asterisk presents Trp112, which was not included in the study. The black bar shows the position of Lys77 involved in monomer aggregation.

biochemically characterized to some extent and have pI values of about 9.2. In contrast to 20 kDa cytolysins they contain cysteine residues and lack tryptophan. The structural features of this group of cytolysins are unknown.

Studies of the thermal stability of an 8 kDa cytolysin from *R. macrodactylus* showed that thermal unfolding of the polypeptide is irreversible (Zhadan and Shnyrov, 1994), and that it combines with phosphatidylcholine but not other membrane phosphatides (Zhadan et al., 1994).

In general, these toxins are not inhibited by sphingomyelin and are substantially less hemolytically active than the 20 kDa and PLA_2-type cytolysins. *T. felina* and *R. macrodactylus* RmI and RmII cytolysins exhibited antihistamine activity (Elliott et al., 1986; Zykova et al., 1998). Cardiotoxicity due to coronary vasoconstriction, resembling that caused by 20 kDa cytolysins, has been demonstrated for the *T. felina* extract IV (Konya and Elliott, 1996). It is not yet known if this effect could be attributed to a 5–8 kDa cytolysin present in that heterogeneous extract.

3.2. Group II

Sphingomyelin-inhibitable 20 kDa cytolysins are the most numerous and extensively studied group of cytolysins produced by sea anemones (Table 2). The name actinoporins has been suggested by Kem (1988). They have recently been given a systematic IUBMB classification as transmembrane solute transporters belonging to the pore-forming equinatoxin family 1.C.38 (Saier, 2000). They are all monomeric, cysteineless proteins with pI values mostly above nine. Three or four toxin molecules oligomerise in the presence of lipid membranes to forms cation-selective pores of ~2 nm hydrodynamic diameter (Turk, 1991; Maček, 1992; Maček et al., 1994). The conductance of rectified single channels in planar lipid membranes produced by toxins of this family range from ~100 to 225 pS in symmetric 0.1 M KCl (Maček, 1992). Chemical modification experiments indicated that the electrical properties of the pores are primarily dictated by an excess of negatively charged amino acid residues, presumably distributed along the ion pathway. Removing the positive charge from lysine residues increased both the conductance and the cation-selectivity of the pore, whereas removing the negative charge from acidic residues had the opposite effect (Belmonte et al., 1993).

Equinatoxins II, IV and V (EqtII, IV, V) from *A. equina* (Anderluh et al., 1996; Pungerčar et al., 1997; Anderluh et al., 1999), sticholysin I (StI) and sticholysin II (StII, formerly known as cytolysin III; Lanio et al., 2001; Blumenthal and Kem, 1983) from *Stichodactyla helianthus* (De los Rios et al., 2000), and cytolysin HMgIII from *Heteractis magnifica* (Wang et al., 2000) have been cloned. In addition, several have been functionally expressed in *E. coli*. It was found that, in addition to the mature protein, the EqtII and HmgIII genes code for a signal peptide followed by a propart bearing an amino acid motif similar to that found in unprocessed sea anemone neurotoxins, nematocyst collagens (Anderluh et al., 2000), and some antimicrobial peptides of the magainin and dermaseptin families (Pungerčar et al., 1997). The propart is composed mainly of polar and negatively charged amino acids and always terminates in a Lys–Arg cleavage sequence for subtilisin-like endopeptidases (Fig. 1). The authors have suggested that the motif might have a role in sorting toxin to the

regulated secretory pathway. Studies also showed that equinatoxins are coded by multiple genes, and that the particular gene lacks introns (Anderluh et al., 1995; Pungerčar et al., 1997).

3.2.1. Structure of actinoporins

Complete or partial amino acid sequences of actinoporins derived either by protein or cDNA sequencing are aligned in Fig. 2. A high degree of internal homology is evident. Also, a higher degree of identity and similarity is evident within either Actiniidae or Stichodactylidae. Similarity searches in protein data bases suggest that actinoporins constitute a unique protein family. A conserved putative N-terminal amphiphilic α-helix, a tryptophan-rich stretch, an RGD-motif (Figs. 1 and 3) are prominent features in the primary structure. Actinoporins are generally considered not to contain carbohydrates. However, in contrast to previous characterization of equinatoxins (Ferlan and Lebez, 1974; Maček and Lebez, 1988), Komatsu et al. (1992) reported the presence of an N-linked oligosaccharide chain in an equinatoxin isoform from *Actinia equina*.

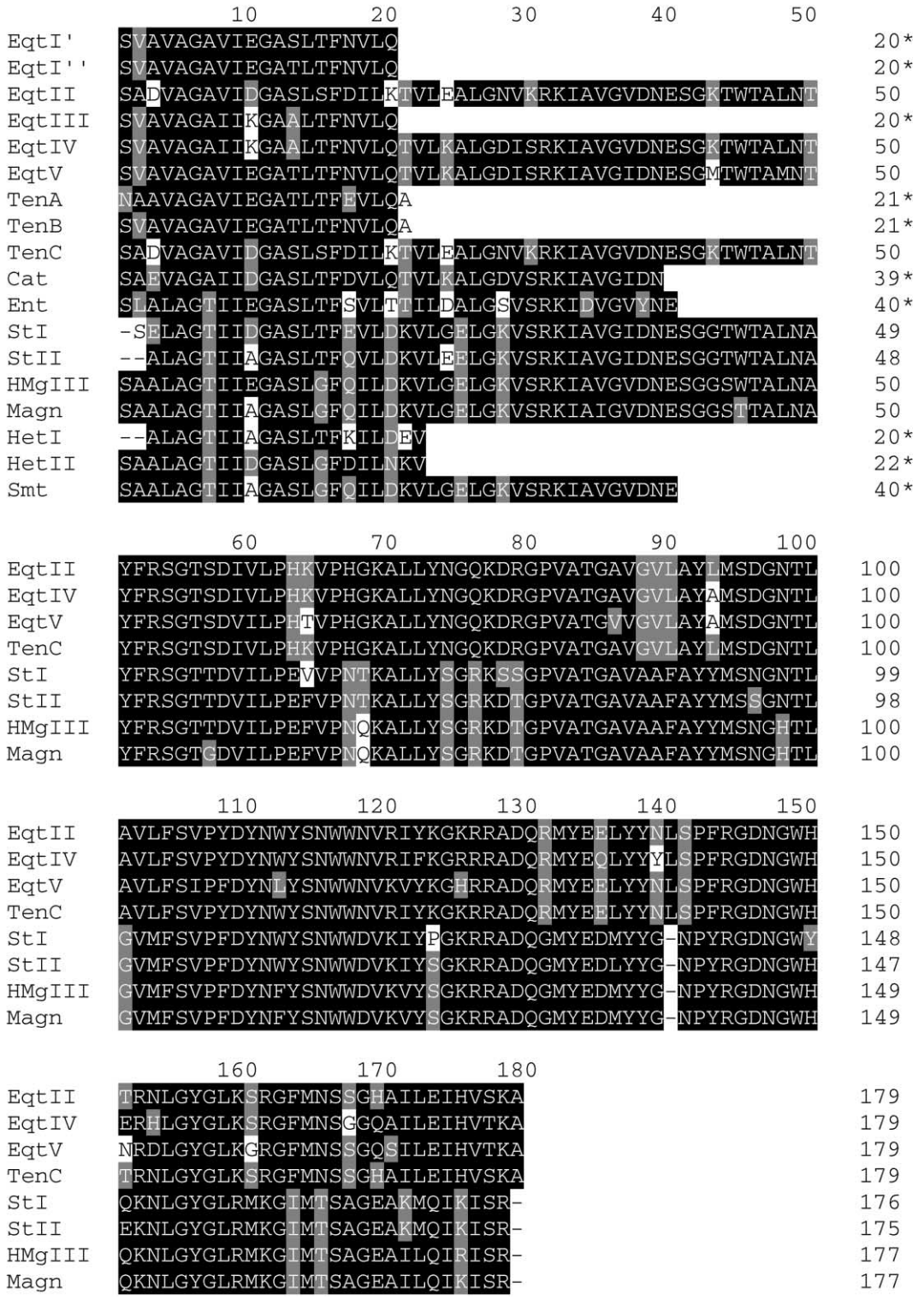
As predicted for *S. helianthus* (Blumenthal and Kem, 1983), *Actinia tenebrosa* (Simpson et al., 1990), and *A. equina* actinoporins, β-structure dominates in all these proteins as indicated by CD- and FTIR-spectroscopy (Belmonte et al., 1994; Menestrina et al., 1999; Poklar et al., 1999; Anderluh et al., 2000). Thus, native StI and StII contain 44–50% β-sheet, 18–20% β-turns, 12–15% α-helix, and 19–22% random coil, which is very similar to the secondary structure composition of EqtII (Menestrina et al., 1999). In contrast, a rather high content of α-helix (37% determined with FTIR-spectroscopy) was reported for EqtII by Caaveiro et al. (2001). This and some previous results interpreted from CD-spectra (Belmonte et al., 1994), however, appear overestimated when compared to the recent 3-D structural data: 10.0% α-helix, 44.7% β-sheet, and 45.3% random coil and β-turns in EqtII (Athanasiadis et al., 2001).

The crystal structure of EqtII resolved to 1.9 Å resolution reveals a tightly folded sandwich of ten β-strands, which is embedded by α-helices A and B (Fig. 3) laying perpendicularly each to other. While α-helix B is fixed to the core of the molecule from both sides, A is free at the N-terminal region and can be displaced without disrupting the general fold of the molecule. A prominent feature of the structure is the extremely tight packing of the side chains in the hydrophobic core of the molecule. The structure further reveals an aromatic patch on the surface of the molecule composed of two tryptophan and four tyrosine residues around the tryptophans 112, 116 and 117 (Figs. 1–3). This part of the molecule is suggested to be inserted in the water-lipid interphase (Maček et al., 1995; Malovrh et al., 2000; Caaveiro et al., 2001; Athanasiadis et al., 2001). The exposed RGD motif may provide additional avidity for certain types of cells as discussed below.

Studies of the secondary structure using FTIR-spectroscopy showed that, upon interaction of StI and StII with sphingomyelin membranes (SM)/phosphatidylcholine (PC) (1:1 molar ratio), the content of α-helix increased only slightly (Menestrina et al., 1999). CD-spectroscopy of EqtII revealed that, on binding to dipalmitoyl-phosphatidylglycerol (DPPG) vesicles, secondary structure was unchanged. However, warming the lipoprotein complex above 60°C increased the EqtII α-helical contents significantly (Poklar et al., 1999). For EqtII, a molten globule-like conformational state induced by either pH, temperature, or chemical denaturants has been observed (Malavašič et al., 1996). It was suggested that the low pH, which may be encountered at the membrane surface, could induce a molten-globule state, a conformation enabling partial unfolding and insertion of the toxin into the lipid bilayer (Poklar et al., 1997). At pH 7–10, pH values promoting actinoporin activity, the existence of a partially folded state, has been demonstrated for sticholysin II and EqtII, which may trigger the pore formation in the plane of membrane (Mancheno et al., 2001; Poklar et al., 2001). These suggestions are in accord with the observation of Khoo et al. (1997). They found that 8-anilino-1-naphthalenesulphonate, a well-known probe of the molten-globule state, stimulates the hemolytic activity of *H. magnifica* cytolysins and EqtII after binding to the vicinity of tryptophan residues. They suggested that a partial conformational change of the toxins might enhance their activity. A transition from a native to a more relaxed conformation of StI, which was induced by pre-incubation of the toxin at a high ionic strength, was suggested to facilitate toxin hemolytic activity (Alvarez et al., 1998). Altogether, these studies suggest that binding of an actinoporin molecule to a membrane correlates with a partial conformational change of the toxin, without significant changes in its secondary structure. At present it is not clear if the observed changes are important for insertion or protein oligomerization.

3.2.2. Interaction of actinoporins with lipid membranes

Since the report of Bernheimer and Avigad (1976), it has been customary to address actinoporins as 'sphingomyelin-inhibitable' cytolysins. Recent studies however have highlighted their lipid preferences for pore-formation in more detail. A study of StII permeabilization of vesicles of different lipid composition demonstrated that pore-formation was maximal either in the presence of 20–25% cholesterol combined with PC or 20–30% SM in PC while vesicles of SM alone were not permeabilized (de los Rios et al., 1998). Similar behaviour was previously reported for StI by Tejuca et al. (1996) who studied the kinetics of adsorption and pore-formation and described a theoretical kinetic model of actinoporin interaction with lipid membranes. The model, in reasonable accord with the experimental data, comprises the following kinetically resolved steps: monomer adsorption to the lipid membrane followed by step-wise association to a trimeric active pore in equilibrium with monomeric and dimeric forms. Moreover, two-dimensional crystals of


10 20 30 40 50
EqtI' SVAVAGAVIEGASLTFNVLQ 20*
EqtI'' SVAVAGAVIEGATLTFNVLQ 20*
EqtII SADVAGAVIDGASLSFDILKTVLEALGNVKRKIAVGVDNESGKTWTALNT 50
EqtIII SVAVAGAIIKGAALTFNVLQ 20*
EqtIV SVAVAGAIIKGAALTFNVLQTVLKALGDISRKIAVGVDNESGKTWTALNT 50
EqtV SVAVAGAVIEGATLTFNVLQTVLKALGDISRKIAVGIDNESGMTWTAMNT 50
TenA NAAVAGAVIEGATLTFEVLQA 21*
TenB SVAVAGAVIEGATLTFNVLQA 21*
TenC SADVAGAVIDGASLSFDILKTVLEALGNVKRKIAVGVDNESGKTWTALNT 50
Cat SAEVAGAIIDGASLTFDVLQTVLKALGDVSRKIAVGIDN 39*
Ent SLALAGTIIEGASLTFSVLTTILDALGSVSRKIDVGVYNE 40*
StI -SELAGTIIDGASLTFEVLDKVLGELGKVSRKIAVGIDNESGGTWTALNA 49
StII --ALAGTIIAGASLTFQVLDKVLEELGKVSRKIAVGIDNESGGTWTALNA 48
HMgIII SAALAGTIIEGASLGFQILDKVLGELGKVSRKIAVGVDNESGGSWTALNA 50
Magn SAALAGTIIAGASLGFQILDKVLGELGKVSRKIAIGVDNESGGSTTALNA 50
HetI --ALAGTIIAGASLTFKILDEV 20*
HetII SAALAGTIIDGASLGFDILNKV 22*
Smt SAALAGTIIAGASLGFQILDKVLGELGKVSRKIAVGVDNE 40*
60 70 80 90 100
EqtII YFRSGTSDIVLPHKVPHGKALLYNGQKDRGPVATGAVGVLAYLMSDGNTL 100
EqtIV YFRSGTSDIVLPHKVPHGKALLYNGQKDRGPVATGAVGVLAYAMSDGNTL 100
EqtV YFRSGTSDVILPHTVPHGKALLYNGQKDRGPVATGVVGVLAYAMSDGNTL 100
TenC YFRSGTSDIVLPHKVPHGKALLYNGQKDRGPVATGAVGVLAYLMSDGNTL 100
StI YFRSGTTDVILPEVVPNTKALLYSGRKSSGPVATGAVAAFAYYMSNGNTL 99
StII YFRSGTTDVILPEFVPNTKALLYSGRKDTGPVATGAVAAFAYYMSSGNTL 98
HMgIII YFRSGTTDVILPEFVPNQKALLYSGRKDTGPVATGAVAAFAYYMSNGHTL 100
Magn YFRSGTGDVILPEFVPNQKALLYSGRKDTGPVATGAVAAFAYYMSNGHTL 100
110 120 130 140 150
EqtII AVLFSVPYDYNWYSNWWNVRIYKGKRRADQRMYEELYYNLSPFRGDNGWH 150
EqtIV AVLFSVPYDYNWYSNWWNVRIFKGRRRADQRMYEQLYYYLSPFRGDNGWH 150
EqtV AVLFSIPFDYNLYSNWWNVKVYKGHRRADQRMYEELYYNLSPFRGDNGWH 150
TenC AVLFSVPYDYNWYSNWWNVRIYKGKRRADQRMYEELYYNLSPFRGDNGWH 150
StI GVMFSVPFDYNWYSNWWDVKIYPGKRRADQGMYEDMYYG-NPYRGDNGWY 148
StII GVMFSVPFDYNWYSNWWDVKIYSGKRRADQGMYEDLYYG-NPYRGDNGWH 147
HMgIII GVMFSVPFDYNFYSNWWDVKVYSGKRRADQGMYEDMYYG-NPYRGDNGWH 149
Magn GVMFSVPFDYNFYSNWWDVKVYSGKRRADQGMYEDMYYG-NPYRGDNGWH 149
160 170 180
EqtII TRNLGYGLKSRGFMNSSGHAILEIHVSKA 179
EqtIV ERHLGYGLKSRGFMNSGGQAILEIHVTKA 179
EqtV NRDLGYGLKGRGFMNSSGQSILEIHVTKA 179
TenC TRNLGYGLKSRGFMNSSGHAILEIHVSKA 179
StI QKNLGYGLRMKGIMTSAGEAKMQIKISR- 176
StII EKNLGYGLRMKGIMTSAGEAKMQIKISR- 175
HMgIII QKNLGYGLRMKGIMTSAGEAILQIRISR- 177
Magn QKNLGYGLRMKGIMTSAGEAILQIKISR- 177

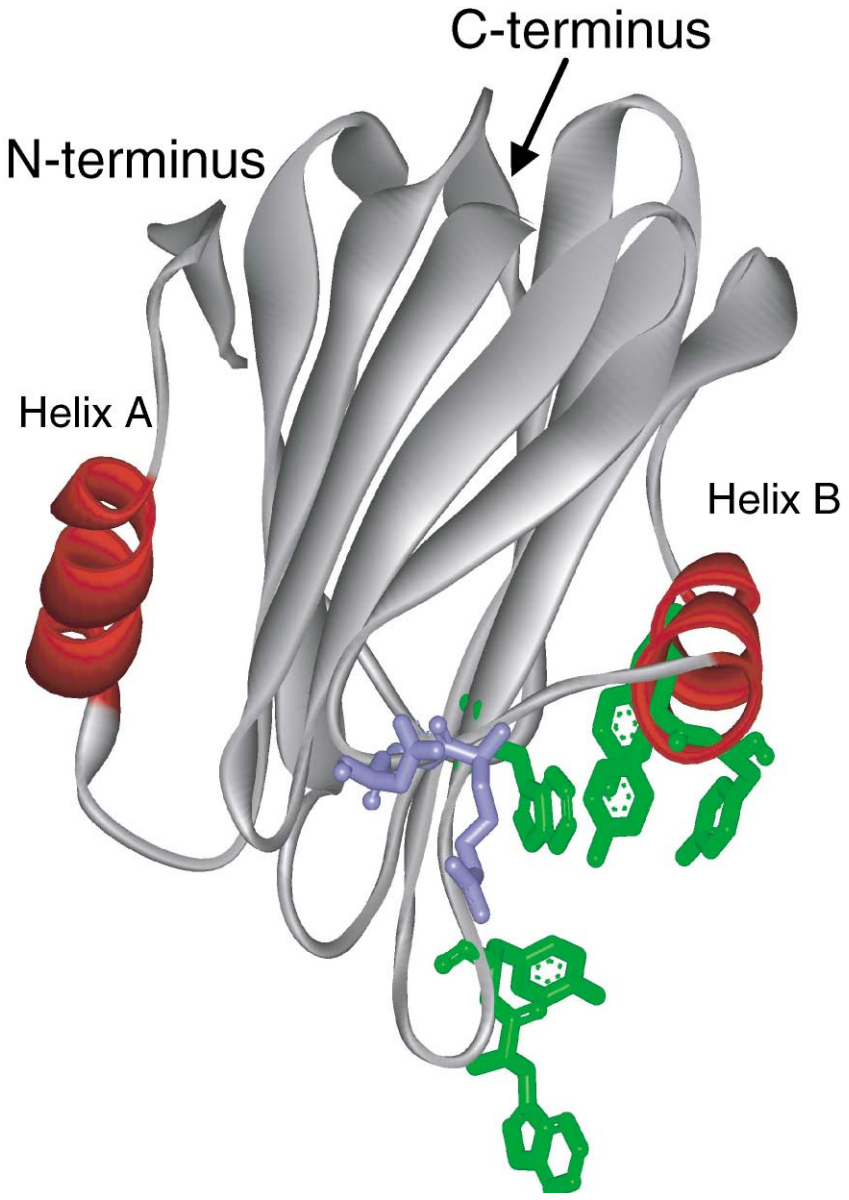


Fig. 3. Three-dimensional structure of equinatoxin II from *Actinia equina*. The ribbon diagram shows structure of EqtII (Athanasiadis et al., 2001). Alpha helices A and B are red; the RGD motif is indicated in blue. Tryptophans residues 112 and 116, and tyrosines residues 113, 133, 137, and 138 form an aromatic patch on the surface of the molecule and are shown green. This patch is considered as seeking for the lipid head group region.

Fig. 2. Alignment of actinoporin amino acid sequences. Sequences are listed for equinatoxins EqtI′, EqtI″', EqtIII (Anderluh et al., 1999), EqtII (GeneBank accession number U41661), EqtIV (AF057028), and EqtV (U51900) from *A. equina*; tenebrosin A (TenA), tenebrosin B (TenB) (Norton et al., 1990), and tenebrosin C (TenC, SwissProt accesion number P17723) from *A. tenebrosa*; caritoxin (Cat) from *Actinia cari* (Belmonte et al., 1994), an actinoporin (Ent) from *Entacmea quadricolor* (Samejima et al., 2000); sticholysin I (StI, P81662), sticholysin II (StII, GeneBank accesion number AJ009931) from *S. helianthus*; magnificalysin III (HMgIII, AF170706), magnificalysin (Magn) (Samejima et al., 2000), actinoporins HetI and HetII from *H. magnifica* (Khoo et al., 1993); and Smt, an actinoporin from *Stichodactyla mertensii* (Samejima et al., 2000). Partial N-terminal sequences are denoted by asterisk, gaps are presented dashed.

StII were recently grown on a PC monolayer and electron microscopy at a resolution of 15 Å revealed tetrameric structures. Actually, these tetrameric structures coincide with the appearance of tetramers in solution (De los Rios et al., 1999), although their significance in relation to functional pores is not clear (Martín-Benito et al., 2000). Interestingly, 2D-crystals of StII could not be obtained on SM or mixed lipid membranes.

EqtII prefers higher concentrations of SM (Belmonte et al., 1993) at least in small unilamellar vesicles (SUV), a fact also observed with StI. In large unilamellar vesicles (LUV), EqtII however shows a preference for equimolar concentrations of SM and PC. Moreover, membranes made of 30% cholesterol in PC were permeabilized by EqtII, but at low temperatures only (Caaveiro et al., 2001). The association of EqtII with PC (Caaveiro et al., 2001), dipalmitoyl-PC and anionic DPPG (Poklar et al., 1999), but without subsequent permeabilization, was demonstrated. Apparent association constants were determined for EqtII binding to SUV made of either SM, SM/egg-PC, or palmitoyl-oleyl-PC and were shown to be 1.42×10^5, 0.51×10^5, and 0.094×10^5 M^{-1}, respectively (Maček et al., 1995). Irreversible EqtII binding to SM/PC (1:1) and reversible binding to pure PC LUVs was characterized by partition coefficients of 2.07×10^5–7.24×10^5 and 8.74×10^4, respectively (Caaveiro et al., 2001).

A clear distinction between the binding and pore-forming steps of EqtII was demonstrated by the dependence of their respective rates on the lipid/toxin ratio: binding has a positive slope, suggesting monomeric binding, whereas pore-formation had a negative slope indicating the requirement for aggregation (Maček et al., 1995). Actinoporin binding to lipid surfaces appears in part to be electrostatically driven, whereas later insertion and oligomerization steps are dependent on the physical state of the lipid bilayer (Shin et al., 1979; Doyle et al., 1989). Recent studies of the chemico-physical properties of the lipid membranes affecting actinoporins binding and pore-formation shed some light on this problem. For example, membrane curvature (Maček et al., 1994), and lipid ordering, membrane dielectric constant and thickness (Maček et al., 1997) have all been suggested to modulate EqtII membrane insertion and oligomerization. Very recently, Alvarez et al. (2001) found that inclusion of phosphatidic acid and other anionic lipids known to induce negative curvature in the lipid bilayer (e.g. cardiolipin, phosphatidylserine, phosphatidylglycerol) into PC:SM LUVs, markedly increased membrane permeabilization by StI and StII. It was suggested that the insertion of toxins could induce formation of a non-lamellar structure contributing to a toroidal pore and also responsible for the increased rate of lipid flip-flop movement.

Site-directed mutagenesis of EqtII has provided insights into its structure–function relationship. It is evident that the actinoporin N-terminal segment, bearing an amphiphilic α–helix A (Figs. 1 and 3), is essential for pore-formation. Gradual truncation by mutagenesis of the N-terminus of EqtII results in substantially decreased hemolytic activity, despite constant binding to erythrocytes (Anderluh et al., 1997). Likewise, fusion of either a longer poly-histidine tag or ubiquitin with the N-terminus of Hmg III from *H. magnifica* impaired the toxin hemolytic activity (Wang et al., 2000). In addition to the N-terminus, a central region of EqtII containing the conserved stretch of clustered aromatic amino acids, WYSNWW, has been suggested to be inserted into the lipid bilayer (Anderluh et al., 1999). Substitution of individual tryptophan residues with phenylalanine showed that, out of five tryptophan residues, only those residing in the central aromatic cluster (Figs. 1 and 3) are essential for both the conformational stability of the molecule and its insertion into lipids (Malovrh et al., 2000). This suggestion is corroborated by chemical modification of aromatic residues, in particular tryptophan (Maček, 1992; Khoo et al., 1997; Campos et al., 1999), which completely abolished EqtII activity. It is also consistent with previous findings that indicated that lipid-bound quenchers were able to quench tryptophan residues when EqtII was inserted into lipid membranes (Maček et al., 1995).

For the purpose of structure–function studies, it is advantageous that wild-type actinoporins lack cysteine. This reactive amino acid can be introduced to a desired position by site-directed mutagenesis. Hence, the molecular topology of the EqtII molecule combined with lipid vesicles has been probed by using single cysteine toxin mutants modified with biotin or fluorescent probes. In one study, cysteine was introduced either at position 77, 126 or 179 and biotinylated. After association with lipid vesicles, all three positions were found to be accessible to avidin in extravesicular solution (Anderluh et al., 1998). Additional topological details were obtained by extensive cysteine-scanning mutagenesis of EqtII combined with labelling of mutants with polarity-sensitive fluorescent probes (Anderluh et al., 1999). At least two regions of the molecule, the N-terminal 13–20 region overlapping the predicted helix and the tryptophan region 105–120 (Figs. 1 and 3), were found to be embedded in the lipid membrane. In EqtII, substitution of lysine 77 by cysteine resulted in decrease of hemolytic activity by two orders of magnitude, despite the toxin conformation remaining intact. This is due to the decreased ability of the mutant to aggregate on lipid membranes (Anderluh et al., 2000a). Pores produced by the mutant, however, exhibited a larger conductance than the wild-type toxin. The hemolytic activity could be recovered almost completely by reintroducing a positive charge at cysteine 77 with bromoethylamine. It was concluded that lysine 77 is involved in toxin oligomerization on membrane.

3.2.3. Pharmacological studies of actinoporins

High lethality of actinoporins in mammals, characterized by an *i.v.* LD_{50} ranging from 20 to 300 μg/kg (Turk, 1991; Maček, 1992), was attributed to cardiorespiratory arrest and coronary vasospasm (Sket et al., 1974; Ho et al., 1987). A

detailed study by Bunc et al. (1999) proved a direct cardiotoxic effect of EqtII on the Langendorff heart preparation at concentrations as low as 0.1–1 nM. Rat heart perfusion with 10 nM EqtII lowered coronary flow by more than 90%, followed by arrhythmia and cardiac arrest. This study also demonstrated that lungs perfused with 100 nM toxin retain up to 99% of the toxin, however the rest in the outflowing perfusate was still vasoconstrictory. Decrease of coronary flow induced by EqtII could be prevented by lowering the extracellular Ca^{2+} concentration (Drevenšek et al., 2000b). The constrictive effect of the toxin was also reduced by 1 μM nicardipine, an L-type calcium channel antagonist (Drevenšek et al., 2000a).

Actinoporins are highly cytotoxic and lytic to a variety of cells and their vesicular organelles. At concentrations less than 0.1 nM, EqtII affected the ultrastructure of V-79-379 cells (Batista et al., 1987). Cells became flattened, lost microvilli, and were covered with blebs, while at the same time, mitochondria swelled, and the Golgi region became vesiculated. It was reported that a lethal dose of EqtII and EqtIII administered to rats, increased plasma concentration of K^+ and glucose (Bunc et al., 1994; Šuput et al., 2001). They also found significant degranulation of granulocytes and platelets. A massive and biphasic K^+-release from erythrocytes, preceding that of hemoglobin, was demonstrated and interpreted in terms of a colloid-osmotic type of cell lysis (Maček et al., 1994), which occurs if induced pores are permeable for smaller solutes but not larger ones such as polypeptides and others. This study also provided evidence that isolated cardioventricular cells were sensitive for EqtII even at concentrations below 10^{-11} M. Nanomolar concentrations of EqtII increased intracellular Ca^{2+} in mouse neuroblastoma (NG108-15) cells with concomitant cell swelling (Meunier et al., 2000).

Little is known about the immunogenic properties of actinoporins. There has been only one study on the humoral and cellular responses to these toxins in mammals. EqtII inhibited by bound lipids was found to elicit production of specific IgG and activation of mice spleenocytes (Narat et al., 1994). It was also demonstrated that EqtII is efficiently inactivated by serum lipoproteins that combine with the toxin and form insoluble aggregates.

Actinoporins have also been reported to interfere with synaptosomal release or uptake of neurotransmitters or their derivatives. Magnificalysins from *H. magnifica* and EqtII were reported to affect GABA and choline uptake (Khoo et al., 1995). The cytolytic fraction Bc2 from the venom of *Bunodosoa caissarum* induced release of glutamate from rat cortical synaptosomes (Migues et al., 1999). It is not clear, however, if the modification of neurotransmitter transport is specific or results from the pore-forming activity of actinoporins in synaptosomal membranes.

Being extremely cytolytic and cytotoxic, actinoporins have been employed as the active component of immunotoxins. Avila et al. (1988) first reported the use of sticholysin from *S. helianthus* for this purpose. EqtII was used as a part of a mitotoxin. It was chemically coupled to transferrin and the resulting conjugates examined on normal and human tumour cells expressing transferrin receptors (Pederzolli et al., 1995). The hybrid molecules, however, retained significant non-specific lytic activity. In another study, sticholysins StI and II and EqtII were explored as antiparasitic agents (Tejuca et al., 1999). The parasitic protozoan *Giardia lamblia* (syn. *duodenalis*) was found to be highly sensitive to actinoporins. Specific targeting of the parasite was approached by a sandwich technique. A high affinity biotin-avidin system was used to concentrate the biotinylated toxin on the parasite surface pre-treated with primary anti-*lamblia* antibodies and secondary avidin-conjugated antibodies. Such treatment increased specificity of targeting the parasite cells four-fold, although considerable non-specific activity still persisted.

3.3. Group III

Cytolytic phospholipases A_2 were first detected in the nematocyst venom of *Aiptasia pallida* (Hessinger and Lenhoff, 1973; Hessinger, 1974; see also review of Hessinger (1988)). At least three hemolytically synergic proteins have been recently isolated from the nematocyst venom of *A. pallida* (Grotendorst and Hessinger, 1999). One of them was identified as a phospholipase A_2. The proteins appear in two isozymic forms. Form α has a m.w. of 45 kDa and pI of 8.8 and form β, 43 kDa. Pure β phospholipase A_2 (β PLA_2; EC 3.1.1.4) is a single-chained glycoprotein that contributes up to 80% of the overall phospholipase activity of the venom. In addition, a non-enzymatic co-lytic factor with a m.w. of 98 kDa, essential for the hemolytic activity of the venom, was purified and characterized. It was assayed by reversing fatty acid inhibition of the venom hemolytic activity. Enzymatic properties of the *A. pallida* β PLA_2 were characterized in more detail (Grotendorst and Hessinger, 2000). This enzyme is activated by Ca^{2+} or Mg^{2+} and its pH-optimum is at 7.7 when assayed on DPPC. It was suggested that the enzyme, with a lysine residue in the active site, 'melts' both fatty acid chains of the substrate.

In addition to these cytolytic phospholipases, isolation and characterization of a novel cytolysin from the sea anemone *Urticina piscivora*, structurally classified as a phospholipase A_2-like type, has been reported. A cytotoxic and cytolytic protein with a m.w. of ~28 kDa and pI > 9.4 was isolated from *U. piscivora* (Cline et al., 1995b). Based on N-terminal amino sequence analysis (Fig. 4), it was reported to be similar to phospholipase A_2-like bungarotoxins from the snake *Bungarus multicinctus.* Like actinoporins, it is a very potent hemolysin devoid of enzymatic activity. It causes hemolysis of rat, guinea pig, dog, pig and washed human red blood cells at concentrations as low as 10^{-10} M. Interestingly, its hemolytic activity is inhibited by sphingomyelin but not by cholesterol, in common with actinoporins. In erythrocyte membranes,

```
                    10        20        30        40
UpI       DENENLYGPNENKAKAKDLTAGASYLTKEAGCTKLQAGCT

UpII      ATDKWNDCGSVTALCEGQKGRNKAT-
UpIII     -DDDWDECGHVTALLEGQQGRNKAAC
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Fig. 4. Partial N-terminal sequences of *Urticina piscivora* cytolysin UpI and cardiostimulating proteins UpII and UpIII

UpI caused numerous cell membrane ruptures as seen by electron microscopy (Cline, 1997). Apart from the hemolytic action, UpI exerted cytotoxicity to different cell lines. HEL299 cells, derived from human embryonic lung, were the most sensitive ($EC_{50} = 30$ μg/ml).

Assayed on guinea pig atrial tissues, 10^{-12} M UpI exerted a positive inotropic effect (Cline, 1997). Experimental animals died from respiratory arrest. UpI also caused haemorrhage and necrosis by dilation of the blood vessels in the skin, and vascular leakage of fluids and rupture of alveolar walls of the lungs. UpI has been demonstrated to be a potent ichtyotoxin, suggesting its role in a predator–prey relationship, since this sea anemone feeds on small fishes.

3.4. Group IV

Currently, metridiolysin, an 80 kDa hemolytic and lethal protein from *M. senile*, is the only representative of this putative group. It was isolated from sea anemone homogenate as a cholesterol-inhibitable cytolysin (Bernheimer and Avigad, 1978). Similarly to a group of bacterial toxins, metridiolysin was found to be activated by thiols and to produce ring structures on membranes (Bernheimer et al., 1979). The toxin exhibits preference for lysing horse and dog erythrocytes, which was not correlated with cholesterol contents in erythrocyte membranes (Bernheimer, 1990).

The action of metridiolysin has also been studied on natural and model lipid membranes (Monastyrnaya et al., 1988). In contrast to the study of Bernheimer et al. (1979), the authors reported a rather unspecific requirement of metridiolysin for lipids. They also demonstrated that the toxin opened fluctuating pores in lipid planar membranes permeable for K^+. They found an asymmetric current-voltage dependence of the pores as also reported for actinoporins that experience asymmetric distribution of fixed charges along the pore lumen (Maček, 1992).

4. Prospects

Since the first reports on the purification and biochemical characterisation of cytolytic toxins from sea anemones (Blanquet, 1968; Hessinger and Lenhoff, 1973; Ferlan and Lebez, 1974; Devlin, 1974), interest in sea anemone toxins has continued to grow. The accumulated data show that these animals produce at least four different classes of cytolytic toxins. In particular those of 5–8 and 80 kDa call for further purification and characterisation. Screening of sea anemones for new toxins (Mahnir et al., 1993) may lead to completion of known toxin groups or even discovery of novel toxin types. Interestingly, Gaphurov et al. (1999) reported that alkaline DNAse purified from *R. macrodactylus* is hemolytic. It is also notable that most of the toxins described so far belong to only four groups of sea anemones, and moreover, that actinoporins are the most numerous.

It has been suggested that sea anemone cytolysins, apart from being efficient weapons for killing prey, may serve as repellents against predators as found for some other pore-forming toxins (Maček, 1992; Mebs, 1994). Both the observed presence of coelenterolysin in gastrocoelomic fluid and release of cytolysins in water support this suggestion. A firm conclusion, however, needs further experimental work.

Concerning the mechanism of action of non-enzymatic cytolysins, it is still unclear whether specific receptors, or some other mechanism, may play a role in addition to a simple lipid binding and formation of pores. For example, erythrocytes and cardiac cells are very sensitive to EqtII even when applied at picomolar concentrations (Maček et al., 1994), in contrast to the rather low association constants estimated for lipid binding to model membranes. The presence of a conserved RGD-motif in all known actinoporin amino acid sequences is also intriguing. At present it is not known whether this motif, found in several proteins interacting with cell membranes, plays a role in toxin action. Either specific receptors, or interference with a particular transduction pathway, may be a cause for the observed pharmacological effects at low doses. For example, intracellular increases in Ca^{2+} caused by actinoporins (Zorec et al., 1990) may produce a variety of cell responses. In such cases, depending on the toxin dose, ensuing events may range from induction of cell proliferation to necrotic or apoptotic cell death. Indeed, Ales et al. (2000) have provided evidence that Bc2, an actinoporin from *Bunodosoma caissarum*, induces a massive exocytosis of catecholamines from bovine chromaffin cells at subnanomolar concentrations, similar to the action of latrotoxin.

Despite the accumulated data, the molecular mechanism of interaction of actinoporins with lipid bilayers is not clear. In this respect, both the recent determination of the crystal structure and progress toward the elucidation of the solution structure of equinatoxin II (Zhang et al., 2000)

will stimulate further mechanistic studies. The conformational structure of the cytolysins, both in free and bound forms, is also a prerequisite for the construction of more efficient immuno- or mitotoxins, acting specifically on targeted cell membranes.

Acknowledgements

The authors thank Dr Gianfranco Menestrina (CNR-ITC Trento, Italy) and Dr Roger Pain (Institute Jožef Stefan, Ljubljana, Slovenia) for revising and commenting on the manuscript. The authors were supported by the Ministry of Education, Science and Sport of Republic of Slovenia.

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