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In vivo effects of cnidarian toxins and venoms

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ABSTRACT

Cnidarians (Coelenterates), a very old and diverse animal phylum, possess a wide variety of biologically active substances that can be considered as toxins. Anthozoan toxins can be classified into two chemically very different groups, namely polypeptide toxins isolated from sea anemones and diterpenes isolated from octocorals. Cubozoan and scyphozoan protein toxins have been the most elusive cnidarian toxins to investigate – despite a tremendous effort in the past few decades, very few of these large, relatively unstable protein toxins were isolated, but recently this has been achieved for cubozoan venoms. Hydrozoans mainly contain large proteins with physiological mechanisms of action similar to the sea anemone and jellyfish pore-forming toxins. This article will focus on the *in vivo* physiological effects of cnidarian toxins and venoms; their actions at the cellular level will only be considered to understand their actions at the organ and whole animal levels. An understanding of mechanisms underlying the *in vivo* toxic effects will facilitate the development of more effective treatments of cnidarian envenomations.

1. Introduction

Searching the recent literature for the effects of cnidarian toxins *in vivo* and on isolated organs revealed a striking lack of data as compared to a large body of data from studies at molecular and cellular levels. Many of these studies focused on their neurotoxic effects on ion channels in excitable membranes, which have been extensively reviewed (Benoit, 1998; Diochot et al., 2007; Smith and Blumenthal, 2007; Wanke and Restano-Cassulini, 2007; several papers in this issue). Toxins, tissue extracts and venoms have also been investigated for their possible enzymatic activity. Phospholipases as constituents of cnidarian venoms have been recently described in detail (Nevalainen et al., 2004). Phospholipase A_2 (PLA_2) has been found in Anthozoa, Cubozoa, Hydrozoa and Scyphozoa, but in highest concentrations in the hydrozoan fire coral *Millepora* sp., the anthozoan stony coral *Pocillopora damicornis*, in the anthozoan sea anemone *Adamsia carciniopados* and in the tentacles of the cubozoan box jellyfish *Chironex fleckeri*. Some of the PLA_2s possess haemolytic and cytolytic activities (Nevalainen et al., 2004), but no study of the *in vivo* effects of these phospholipases in mammals has yet been published.

2. Anthozoa

2.1. Sea anemone toxins

Substances isolated from sea anemones have served as useful molecular models or probes in biomedical research and will continue to do so. Sea anemone and hydrozoan extracts were used by Portier and Richet to unravel some important and general aspects of the phenomenon of anaphylaxis (see initial article by the editors). The sodium channel modulating peptides were originally studied as putative cardiac inotropes and are currently used to investigate cardiac arrhythmias. Peptides blocking potassium and other ion channels are described by others in this issue.

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Another ubiquitous and well-studied class of sea anemone toxins are the cytolytic polypeptides (Reviews: Maček et al., 1994; Bernheimer, 1996). There are four known groups of cytolysins, based on their differing molecular properties and modes of action. The first group comprises small cysteine containing 5–8 kDa peptides that bind to phosphatidylcholine in cellular membranes and form pores; it also has been shown that they also can inhibit histamine-induced contractions of the guinea-pig ileum (Aldeen et al., 1981). The second group consists of 20 kDa proteins with isoelectric points above 9, and are referred to as actinoporins (Kem, 1988). Sphingomyelin is the membrane lipid acceptor for these cytolysins, which are stable proteins devoid of cysteine and form cation selective pores by oligomerization within the membrane. The third group comprises heterogeneous 28–45 kDa proteins which may or may not possess phospholipase A2 (PLA2) activity (Cline et al., 1995; Grotendorst and Hessinger, 2000). A member of this group, Up I, isolated from *Urticina piscivora*, was haemolytic and cytolytic to several tumour cells. Its cytolytic action can be inhibited by sphingomyelin (Cline et al., 1995; for a related Urticina cyltolysin, see Razpotnik et al., 2009). A cytolysin from *Metridium senile* is the only known member of the fourth group. It is a lethal 80 kDa protein whose haemolytic activity is inhibited by cholesterol (Bernheimer et al., 1979). Several recent reviews on cytolytic toxins from sea anemones have been published describing the structure (Anderluh and Macek, 2002; Kristan et al., 2009; Alvarez et al., 2009) and mechanisms of channel formation by actinoporins in lipid membranes (Alegre-Cebollada et al., 2007).

While most toxins produced by sea anemones are polypeptides, other toxic substances have also been found. Interestingly, palytoxin, first isolated from Zoanthids (Palythoa), has also been found in the sea anemone *Radianthus macrodactylus* (Mahnir et al., 1992). Palytoxin is one of the most potent known toxins, and its *in vivo* effects in several mammals, including man, include neurotoxicity, rhabdomyolysis, and cardiovascular collapse (Hoffmann et al., 2008). Another toxin, caissarone, is an iminopurine isolated and purified from the sea anemone *Bunodosoma caissarum*. It has been shown that the increased intestinal motility observed in experimental animals after administration of caissarone is due to its competitive antagonism at an adenosine receptor (Cooper et al., 1995). A crude extract from *B. caissarum* caused convulsions in mice. It seems that the convulsions are not caused by a putative presence of a cytolysin in the venom or by an increase in glutamate release. The experimental data suggest that a peptide toxin directly interacting with NMDA receptors may cause this effect (Gondran et al., 2002).

An assessment of the pharmacological actions of sea anemone venom or crude extracts *in vivo* and on isolated organs is missing for most of the described toxins. The fact that several types of toxins may coexist in the venom of a single sea anemone further complicates the study of their effects *in vivo* and *in vitro,* especially when taking into account that it is difficult to isolate undischarged nematocysts, the presumed source of toxins, in quantity sufficient to obtain enough pure venom in most species. Here, *in vivo* and isolated organs effects of purified sea anemone toxins on mammals are presented with emphasis on studies carried out during the last two decades.

2.1.1. *Cellular and* in vivo *effects of sea anemone neurotoxins*

Several sea anemones produce potent neurotoxins. The best known neurotoxins are the sodium channel toxins. All of these toxins prevent inactivation of sodium channels by stabilizing the open state conformations, and their structures (Norton et al., 2004; Norton, 2009) structure–activity properties (Moran et al., 2009) and actions on various mammalian sodium channels (Wanke et al., in press) are discussed in detail in other parts of this issue of Toxicon. Potassium channel blockers from sea anemones, especially the Kv 1(Shaker type channel) blockers and other toxins have become valuable experimental tools and their immunosupressive actions (Kalman, 1998; Pennington, 2009) may be the basis for their drug development. Reversible block of potassium current by a sea anemone toxin was first described for equinatoxin from *Actinia equina*; it reversibly decreased potassium current in single myelinated nerve fibres from frog (Šuput et al., 1986). Later it was shown that *A. equina* contains a smaller peptide, a ShK like channel blocker (Minagawa et al., 1998). Therefore, it is possible that the previously described effect of equinatoxin on the potassium current was due to the contamination of equinatoxin with this smaller peptide. So far several well-defined potassium channel toxins from a number of sea anemones have been isolated and well characterized, which is reviewed elsewhere in this volume (Castenada and Harvey, in press). An inhibitor of ether-a-go-go-related gene potassium channels APETx1, has also been isolated from *Anthopleura elegantissima* (Diochot et al., 2003; Wanke and Restano-Cassulini, 2007). APETx2 from the same sea anemone is a blocker of acid-sensing ion channels (Diochot et al., 2007), which are permeable to several cations.

Granulitoxin (GRX) is a lethal (LD_{50} 400 μg/kg) neurotoxic ≈5 kDa peptide isolated from *Bunodosoma granulifera*. Intraperitoneal (i.p.) injection of the toxin in mice caused circular movements, aggressive behaviour, convulsions and death (Santana et al., 1998). Intrahippocampal injection in rats of granulitoxin caused seizure-type brain activity that began in the hippocampus and spread rapidly to the occipital cortex, as shown by EEG measurements. During that period akinesia presented as the most prominent symptom, but then facial automatisms, head tremor, salivation, rearing, jumping, barrel-rolling, wet dog shakes and forelimb clonic movements were also observed. Those effects developed further into the status epilepticus, and eventually the experimental animals died (Santana et al., 2001).

2.1.2. *Sea anemone toxins acting on the cardiovascular system*

Many sea anemone toxins including cytolysins and neurotoxins have been investigated for their putative cardiotonic actions. A positive inotropic effect (increase in contractile force of myocardium) has been described for most purified sea anemone neurotoxins that have been tested. Unfortunately, this potentially useful effect is transient and is rapidly followed by cardiotoxic effects that

include arrhythmias (triggered by early after depolarizations resulting from incomplete sodium channel inactivation) and systolic arrest due to myocardial cell calcium ion overloading.

Cardiostimulant effects of anthopleurins have been described (Shibata et al., 1976): it was proposed that the toxins might be used as cardiotonic drugs (Gross et al., 1985). Five toxins (APE 1–APE 5) isolated from *A. elegantissima* are neurotoxins, but APE 1-1, APE 2-1 and APE 5-3 also produced a positive inotropic effect in mammalian heart muscle. Threshold concentrations for the effect were as low as 1 nM. The isotoxins had also a positive chronotropic effect in the isolated guinea pig right atrium (Bruhn et al., 2001). A possible therapeutic effect of a recombinant neurotoxin hk2a from the sea anemone *Anthopleura* sp. has been recently studied with a canine model of acute cardiac insufficiency caused by rapid ventricular pacing, which causes a drop in the left ventricular ejection fraction (LVEF). Intravenous injection of recombinant hk2a into a dog caused a rapid and sustained increase in LVEF without significant effect on the heart rate. It was argued that the recombinant hk2a is more effective than digitalis, a glycoside inhibitor of Na^+/K^+ pump, in the treatment of acute myocardial insufficiency (Ouyang et al., 2005).

A curious effect on rat blood pressure has been reported for a crude extract from *Bunodosoma cavernata*. At low intravenous doses up to 8 μg protein/kg, the extract caused a transient hypotension, but at higher doses the transient hypotension was followed by a sustained and dose-dependent hypertension (Eno et al., 2001). The hypotension was resistant to any treatment, but hypertension could be prevented by application of the β-adrenergic antagonist propranolol. Bradycardia was also present, especially in doses above 20 μg protein/kg. Because the long sea anemone neurotoxins affect both neuronal and myocardial sodium channels, some of these cardiovascular effects may be due to the effects on autonomic neurons and ganglia. Further investigations with purified venom constituents are required to determine which actions are due to a particular toxin.

2.1.3. Actinoporins

Actinoporins are basic proteins with m.w. ≈ 20 kDa that interact with sphingomyelin in cellular and artificial membranes, and share a common property that they bind to phospholipid domains of membranes, oligomerize and form cation selective pores. Interactions of actinoporins with membranes are thoroughly described for equinatoxins (Kristan et al., 2009) and sticholysins (Alvarez et al., 2009) in this special issue of Toxicon. Here the pharmacological effects of actinoporins are described using equinatoxins II and III as examples.

Before the isolation of the three known isotoxins (EqT I-III), a toxin named "equinatoxin" (EqT) was used in all the studies. EqT roughly corresponds to the abundant EqT II, but the other two isotoxins might have also been present. In older experiments perfusion of isolated rat lungs with 80–200 ng EqT resulted in a dose-dependent increase in fresh weight of the perfused lungs that was caused by an increase in vascular permeability (Lafranconi et al., 1984). An increased perfusion pressure was also observed after application of EqT. The effect was explained by a putative release of arachidonic acid from the treated tissue and by subsequent formation of prostaglandins, but this hypothesis has never been proven or corroborated with further experiments. A positive inotropic action of EqT on guinea-pig atrium has been described (Ho et al., 1987), and the effect could be diminished by the application of indomethacin, a cyclooxygenase inhibitor. A positive inotropic effect of EqT II has also been observed for the isolated guinea-pig heart at sub-nanomolar concentrations. Higher concentrations of EqT II caused an irreversible drop in coronary perfusion, resulting in a decreased myocardial contractility (Budihna et al., 1990). It has been shown that EqT II from *A. equina* and the very similar tenebrosin-C from *Actinia tenebrosa* are structurally identical toxins with similar cardiac stimulatory action (Norton et al., 1992), and the finding stimulated a discussion about the taxonomic relationship between the two species of sea anemones. Although a transient "cardiotonic" effect of EqT was a regular finding, the animals died due to an as yet unexplained cardio-respiratory arrest.

The cardinal *in vivo* actinoporin signs after an i.v. application of $3 \times LD_{50}$ of any equinatoxin are cessation of breathing, hypotension, arrhythmia and, finally, a complete cardiovascular collapse. Initially the cardiotoxic mechanism of EqT II was unknown. It was not clear whether the toxin, injected i.v. into an experimental animal, reached the heart in a concentration sufficient to cause direct effects on the heart, or the heart failure was secondary to an increased resistance in the pulmonary circulation This issue was resolved by performing simultaneous experiments on rats, *in vivo* on isolated rat hearts, and on isolated rat lungs. The lungs were perfused with a calculated "lethal" concentration of EqT II and the perfusate was collected in order to determine the residual concentration of EqT II, which was found to range from 0.8 to 5 nM. The hearts were perfused with different concentrations of the toxin and with the effluent from the lungs collected during perfusion of the lungs with EqT II. There were direct, dose-dependent cardiotoxic effects of the toxin and of the effluent from the lungs on Langendorff heart preparations. The threshold concentration of EqT II causing a drop in the perfusion rate, decrease in left ventricular pressure, arrhythmia and increased LDH release, was found to be around 0.1–1 nM. With 10 nM EqT II the left ventricular pressure dropped to $14 \pm 11\%$ of the control, and the coronary flow to $9 \pm 3\%$. These effects were followed by arrhythmia and cardiac arrest (Bunc et al., 1999a). However, these experiments could not assess the possible involvement of haemolysis and hyperkalemia in the lethal effects of the toxin. EqT II is an actinoporin, therefore it is haemolytic. Release of K^+ from erythrocytes could be directly cardiotoxic, so another set of experiments was designed to settle this question. Respiratory activity, ECG and animal serum ionic composition were monitored in anesthetised rats after i.v. application of a lethal dose of EqT II. First K^+ concentration was measured in dead animals after the experiment. In the second experiment hyperkalemia was simulated by an i.v. injection of KCl solution in an amount sufficient to reach the final concentration of K^+ in the plasma as observed after the lethal intoxication with EqT II. KCl, at

concentrations detected in serum of rats killed by the toxin, caused only a transient arrhythmia, but did not cause the death of experimental animals, suggesting that the direct cardiotoxic effects were the sole cause of death of experimental animals (Bunc et al., 2000a). Similar experiments were performed comparing the effects of EqT III with the effects of hyperkalemia caused by the injection of KCl giving the same final concentration of K^+ in the plasma as that observed after an i.v. injection of a $3 \times LD_{50}$ dose of EqT III. Again, artificial hyperkalemia alone was not lethal (Šuput et al., 2001). That cardiotoxicity is the principal cause of the lethal effect of these toxins is also supported by the observation that EqII is more toxic ($LD_{50} = 35$ μg/kg), but less haemolytic than EqT III ($LD_{50} = 83$ μg/kg). Nevertheless, the exact mechanism of Eq cardiotoxicity remains unknown.

Vasoconstriction may be an important mechanism of the cardiotoxic action of EqT II. It has been shown that EqT II binds to vascular smooth muscle membranes, decreasing membrane fluidity and increasing the resting conductance (Šentjurc et al., 1996). Direct measurements of the tension of coronary smooth muscle were made on isolated porcine coronary arteries using of 1–100 nM EqT III. The toxin caused a slight increase in the tension of the coronary artery rings and also augmented the KCl-induced vasoconstriction *in vivo* (Šuput et al., 2001). It is known that actinoporins form cation selective pores in cellular membranes, and this leads to an increase in intracellular Ca^{2+}concentration. The role of extracellular Ca^{2+} in the cardiotoxic effects of EqT II has been confirmed using isolated rat hearts. Although perfusion of rat hearts with EqII dissolved in a Ca^{2+} free solution had no effect on coronary flow, a maximal decrease in coronary flow was reached with EqT II dissolved in solution with 1.5 mM Ca^{2+} (Drevensek et al., 2000).

It is reasonable to assume that i.v administered EqT II initially binds to endothelial cells. This view is supported by previous data showing that EqT binds to lungs (Bunc et al., 1999a; Bunc et al., 1999b). Vasoconstriction can, therefore, be mediated by endothelins released from the affected endothelial cells. Porcine coronary arteries with intact endothelium were exposed either to endothelin-1 or to EqT II. It was shown that endothelin-1 is at least three times as potent as EqT II in increasing the vascular smooth muscle tension. Tezosentan, an endothelin inhibitor, reduced both ET-1 and, to a lesser extent, EqT II-induced contractions of the isolated porcine coronary artery. This suggests the possibility that EqT II-induced vasoconstriction is caused by a combination of direct and indirect, endothelin-1 dependent effects (Drevensek et al., 2002).

Other mechanisms might be involved in the respiratory arrest resulting from i.v. application of EqT II. The cessation of respiration could be due either to an increased resistance to blood flow and consequent decreased compliance of the lungs, or it could be caused by a direct action on the respiratory centre located in the medulla oblongata, assuming that the toxin has access to this region of the brain. The latter should be considered as a serious option, as it has been shown that EqII causes swelling and lysis of differentiated neuroblastoma (NG108-15) cells, provided Ca^{2+} is present in the medium (Meunier et al., 2000). Similarly, a calcium-dependent swelling of the nerve membrane in the node of Ranvier has also been observed after application of EqT II. Further studies are necessary to understand the mechanism underlying the cessation of breathing after i.v. administration of EqT II.

2.2. Octocoral toxins

Soft corals produce a diverse group of biologically active substances, including polypeptides, diterpenes (Berrue and Kerr, 2009), palytoxin (Wu, in press), and molecules related to certain pigments, i.e., zooxanthins, parazoanthoxanthins etc. (Cariello and Tota, 1974; Turk et al., 1995; Strupi Šuput et al., 1996). This diverse chemistry has generated a variety of biological activities: cytotoxic, cardiotonic, cardiotoxic, neurotoxic, anti-inflammatory, anti-viral and anti-neoplastic. The diversity of octocoral toxins will become more apparent in the review by Ferchmin (Ferchmin et al., in press).

Toxins interfering with cholinergic neurotransmission have been found in soft corals. Lophotoxin, a diterpene lactone isolated from several gorgonian corals, irreversibly blocks cholinergic transmission by binding to nicotinic cholinergic receptors (Abramson et al., 1988; Sorenson et al., 1987). Stolonidiol, a bioactive marine diterpenoid from the Japanese soft coral *Clavularia* sp., has been shown to induce choline acetyl transferase (ChAT) activity in cultured cholinergic neurons. The authors suggested that it might act as a neurotrophic factor-like agent on the cholinergic nervous system (Yabe et al., 2000).

An ethanolic extract from *Parazoanthus axinellae* was lethal to crabs and mice; atropine reduced its lethality in mice (Strupi Šuput et al., 1996). Further investigations revealed that the extract contained substances which inhibited acetylcholinesterase (AChE) activity. Their isolation yielded several AChE inhibitors. The most abundant inhibitor was identified as pseudozoanthoxanthin, which is chemically related to tetrazacyclopentazulene natural pigments found in the following genera: *Parazoanthus*, *Epizoanthus*, *Zoanthus* and *Palythoa*. Pseudozoanthoxanthin is a competitive inhibitor of AChE with a Ki of 4 μM. A detailed study showed that, *in vivo,* both the crude extract and the isolated inhibitor cause a picture typical of systemic AChE inhibition. Obstruction of respiration was seen as an increased residual volume, and the expiration time was significantly prolonged. Simultaneously hypotension and a marked bradycardia developed, but the latter effect was occasionally replaced by tachycardia, which could be explained by activation of baroreceptor reflexes and/or by indirect stimulation of pre-ganglionary nerves in the sympathetic nervous system (Turk et al., 1995; Strupi Šuput et al., 1996).

That analgesic and anti-inflammatory substances are some of the most widely used drugs provided an added impetus to search for substances with these activities in soft corals. The gorgonian *Pseudopterogorgia elisabethae* produces two glycosides, pseudopterosins A and E, which are analgesic *in vivo*. In mice they prevent ear oedema when applied either topically or systematically (Mayer et al., 1998). Anti-inflammatory and analgesic action of lemnalol, a natural compound isolated from the marine soft coral *Lemnalia cervicorni*, has been studied in rats and on LPS stimulated

RAW 264.7 cells *in vitro*. Intramuscular injection of lemnalol into rats prevented carrageenan induced paw oedema in rats; the substance also exhibited analgesic properties. Lemnalol inhibited expression of pro-inflammatory enzymes in the cultured cells (Jean et al., 2008).

Although several toxins have been isolated from soft corals, very few studies on the effects of those toxins have been performed *in vivo*. A prolonged survival of mice with implanted sarcoma and treated with clavulone II and chlorovulone I has been reported (Honda et al., 1988), and prevention of tumour promotion by teleocidin has been observed after treatment of mice with sarcophytols A and B (Fujiki et al., 1989). A search for anti-tumour compounds in fourteen gorgonian octacorals resulted in isolation of eupalmerin, a stable, cytotoxic and antineoplastic compound. The substance activated the mitochondrial pathway of apoptosis in two types of human malignant glioma cells and also has been effective *in vivo* against subcutaneous malignant glioma xenografts. A week of therapy with daily intra-tumour injections of eupalmerin acetate significantly reduced tumour growth; therefore eupalmerin acetate might be considered as a candidate for anticancer therapy (Iwamaru et al., 2007).

Some proteinaceous toxins have also been reported to occur in octocorals. Nematocyst content and partially isolated proteins from nematocysts of Red Sea stinging corals *Dendronephthea* sp, *Nephthea* sp, and *Heteroxenia fuscescens* are lethal to mice and cause haemolysis of human erythrocytes. Extracts from those animals cause dermonecrosis and oedema when injected locally in sublethal concentrations (Radwan et al., 2002). These toxins seem to have pharmacological properties characteristic of cytotoxic and cytolytic proteins.

3. Cubozoa

Jellyfish, especially the box jellyfish, are dangerous, very toxic marine animals; many deadly human contacts with them have been described in the medical literature. One of the first publications was a description of an account of a skin lesion after a contact with a jellyfish *Cyanea capillata* (Kristenson, 1949). A publication of a severe envenomation by the pelagic hydrozoan *Physalia physalis* (Klein and Bradshaw, 1951), and a description of serious, even fatal stings by jellyfish from Northern Queensland (Flecker et al., 1952a) followed shortly after that article. Since then a number of attempts have been made to isolate lethal factors from jellyfish, but only with limited success. Most venom components seem to be very labile and different methods of venom collection and active compound isolation have made interpretation of the results difficult. Despite the elusive nature of toxic components, several toxins have recently been isolated and cDNAs encoding those toxins have been sequenced (See paper by Brinkman and Burnell, 2009). Some detailed reviews of coelenterate venom research (Burnett and Calton, 1987; Tidballs, 2006) have been published that include comprehensive descriptions of jellyfish biology, toxin biochemistry, and envenomation therapy. Still, our knowledge of jellyfish toxins is still relatively superficial and current therapeutic approaches are only symptomatic and empirical (Bailey et al., 2003; Little, 2008). Here the known physiological effects of jellyfish toxins are described, and compared with those of other cnidarian toxins.

3.1. Effects of cubozoan toxins

3.1.1. Chironex fleckeri *(Box jellyfish, Sea wasp)*

Owing to its potentially lethal venom, this dangerous jellyfish is one of the most feared animals in the world. Initial research demonstrated that the venom was lethal to crabs and mice. Sublethal doses were neurotoxic in crabs. Experiments with mice and rats showed that i.v. application of the venom caused a rapid cessation of respiration followed by cardiac arrest (Endean et al., 1969; Freeman and Turner, 1969), which was similar to its lethal effects on humans. It seems that in addition to the lethal component there may also be other components present in the venom that are responsible for its neurotoxic, haemolytic/cytolytic, myotoxic, and dermatonecrotic activities. The recent cloning of two protein components offers a promising new approach for the investigation of the various constituents of the venom (Brinkman and Burnell, 2007).

Analysis of the lethal components is most important for limiting the potentially fatal effects of *C. fleckeri* stings in humans. Although these components have not yet been unequivocally identified, all lethal fractions produced the same effects after an i.v. injection (Mustafa et al., 1995; Ramasamy et al., 2004). Initial rise of the blood pressure (BPa) was followed by a profound hypotension. Bradycardia was a prominent finding and within minutes AV block developed. A marked vasoconstriction was observed and the heart stopped in systole. Respiratory arrest was also a regular finding, and it was believed to be of CNS origin. Experimental animals survived administration of large intraperitoneal, subcutaneous and intradermal doses of *C. fleckeri* venom. The effects of the venom from *C. fleckeri* were remarkably similar to the effects of the previously described equinatoxins *in vivo* and on the isolated rat heart (Bunc et al., 1999a; Bunc et al., 2000a; Drevensek et al., 2000; Šuput et al., 2001), which suggests that membrane permeabilisation and the subsequent entry of sodium and calcium ions into the cells may be an important mechanism of toxin lethality. An increased entry of Na^+ into cardiomyocytes has been observed after application of a toxin isolated from *C. fleckeri*. This was followed by an entry of Ca^{2+}, possibly due to activation of the Na^+/Ca^{2+} exchanger (Mustafa et al., 1995). Novel nematocyst collection methods, coupled with new venom and toxin isolation procedures provided new substances with results after i.v. applications nearly identical with those caused by the less pure fractions used in older experiments. Ramasamy et al. (2005a,b) showed that *C. fleckeri* venom produces biphasic effects on arterial blood pressure and ultimately, circulatory collapse. These effects could not be completely prevented by administration of box jellyfish antivenom, but the prophylactic effect was sufficient to prevent mortality in 40% of the rats. Simultaneous administration of $MgSO_4$ prevented cardiovascular collapse and death in all animals, although it could not prevent the development of a transient

hypotension (Ramasamy et al., 2004). Although venom isolated from purified nematocysts produced only hypotension, extracts from tentacles devoid of nematocysts caused a hypertensive response which could be prevented by prazosin (Ramasamy et al., 2005b). With nematocyst venom cardiovascular collapse was found in two of five treated animals. It has also been shown that *C. fleckeri* venom produces sustained contractions of endothelium-intact as well as endothelin-denuded aorta rings (Winter et al., 2007).

Two toxins form *C. fleckeri* (CfTX-1 and CfTX-2) have been identified, sequenced and cloned (Brinkman and Burnell, 2007); the effects of the recombinant toxins have not yet been published. Recently it has been shown that CfTX-1 and -2 are subunits of a 370 kDa haemolysin, and that another haemolysin with m.w. 145 kDa is composed of another two subunits with m.w. 39 and 41 kDa which do not react with commercially available box jellyfish antivenom or rabbit polyclonal antibodies raised against *C. fleckeri* nematocyst extracts (Brinkman and Burnell, 2008). The authors stress the importance of further *in vivo* and *in vitro* testing of those toxins which may be responsible for the lethal actions of the *C. fleckeri* whole venom.

3.1.2. Chiropsalmus quadrigatus *(Habu-kurage, Fire Medusa)*

Early work done with venom and venom extracts from *C. quadrigatus* reported identical, though less potent effects on experimental animals relative to the toxic substances present in *C. fleckeri* (Freeman and Turner, 1972). Similar effects were observed on victims stung with these jellyfish (Burnett and Calton, 1987). Cardiovascular collapse was also observed three decades later also by Ramasamy et al. (2005a,b), who showed that antivenom against *C. fleckeri* alone or in combination with $MgSO_4$ could not prevent the hypotension and cardiac arrest caused by injection of *C. quadrigatus* venom in experimental animals. The effects of the venom were also resistant to prazosin, ketanserin and artificial respiration (Ramasamy et al., 2005a).

A toxin from Okinawan *C. quadrigatus* (CqTX-A), which is probably a different species from the Australian one (Tibballs, 2006), has been isolated and its cDNA sequenced (Nagai et al., 2002). However, most of the research on experimental animals was done with a less pure toxin from the Okinawan *C. quadrigatus*. It has been shown that the toxin induces Ca^{2+} and endothelium dependent contractions of aortic rings, and increases the tension of rat right atrium (Sakanashi et al., 2002). In rabbits it produced a transient hypertension followed by hypotension and bradycardia. Arterial flow decreased and animals died due to cardiac arrest. These effects could be prevented or diminished by a Ca^{2+} channel blocker (Koyama et al., 2003). Saline extracted venom from nematocysts caused an abrupt onset of hypertension and bradycardia when injected i.v. into anaesthetised rats. Prazosin, atropine and an endothelin receptor antagonist could not prevent the effects of the toxin on rats *in vivo*. Again, a calcium channel blocker (nicardipine) prevented the effects of the toxin and experimental animals survived the treatment (Noguchi et al., 2005).

3.1.3. Carukia barnesi *(Irukandji)*

C. barnesi is probably the best known carybdeid jellyfish, due to the potentially deadly "irukandji" syndrome (Flecker, 1952b) that develops after its sting. Unfortunately, most of the data on its *in vivo* effects have been collected from sting victims. The most prominent and life threatening effect of the venom is malignant hypertension, which may result in acute cardiac failure with pulmonary oedema and/or intra-cerebral haemorrhage (Marshall, 1964; Fenner et al., 1988; Martin and Audley, 1990; Tibballs et al., 2001; Winkel et al., 2005). It is not yet clear whether *C. barnesi* is the only jellyfish that causes irukandji or irukandji-like syndrome (Little et al., 2006). The composition of the venom may change with age and environmental conditions (Underwood and Seymour, 2007). A toxin has yet been isolated from this animal; therefore, it is not surprising that the pharmacology of its venom is poorly understood. It seems that the venom causes a large elevation in blood cateholamines, leading to the above-mentioned effects; i.v. administration of phentolamine or sublingual nitrates can ameliorate or reverse the symptoms (Huynh et al., 2003). Administration of $MgSO_4$ decreased pain and hyperadrenergic symptoms (Tibballs, 2006). As cardiac failure may precede hypertension, a direct cardiotoxic action of the toxin may also be possible, but only a positive inotropic effect on isolated rat and guinea-pig atria has been observed. This has also been described for equinatoxin from *A. equina* (Ho et al., 1987), but later it was shown that the toxin is cardiotoxic (Bunc et al., 1999a; Bunc et al., 2000b; Drevensek et al., 2000). Increased membrane permeability for Na^+ probably triggers the release of catecholamines, as tetrodotoxin (TTX) blocks the tachycardia in isolated rat and guinea-pig atria (Winkel et al., 2005).

The symptoms and clinical course of irukandji syndrome are strikingly different from the effects of *C. fleckeri* stings. This indicates that the toxins produced by these animals are different, although antivenom against *C. fleckeri* toxins binds to venom components from *C. barnesi* (Wiltshire et al., 2000).

3.1.4. Carybdea rastonii *(Andokurage, Sea wasp),* Carybdea alata *(Hawaiian box jellyfish, Sea wasp), and* Carybdea marsupialis *(Carribean box jellyfish)*

Partly purified toxin (pCRTX) isolated from *C. rastonii* induced calcium-dependent contractions of rabbit aorta. Contractions were absent in calcium-free media, or after pre-treatment of aortic strips with Ca^{2+} channel blockers. TTX had no effect on pCrTX induced contractions but bathing the aortic strips in low Na^+ medium reduced contractions significantly (Azuma et al., 1986a). In relatively low concentrations (10^{-8}–10^{-7} g/ml) pCrTX caused contraction of rat aorta and guinea-pig taenia coli, but in higher concentrations (10^{-7}–10^{-6}) it relaxed rat aorta precontracted by norepinephrine. Relaxation was prevented either by methylene blue or by removal of endothelium, which indicated that pCrTX induced relaxation was caused by release of endothelium derived relaxing factor (EDRF) from intact endothelium (Nagase et al., 1987).

It has also been shown that pCrTx is a potent activator of platelets. The concentration which produced 50% aggregation of platelets was 1.8×10^{-7} g/ml for pCrTX and 2.3×10^{-6} g/ml for collagen. The effect of pCrTX was Ca^{2+} dependent, but Ca^{2+} blockers were ineffective in preventing the pCrTX induced platelet aggregation (Azuma et al.,

1986b). It has been proposed that the toxin increased cation permeability indiscriminately thus depolarizing the platelet membrane. Purified toxins CrTX-1, CrTX-II, and CrTX-III obtained from the tentacles of the jellyfish also aggregate platelets by increasing cation permeability permitting an influx of calcium (Azuma et al., 1986b).

Later, a 43 kDa toxin CrTX-A was isolated from the nematocysts, and a 46 kDa CrTX-B from the tentacles of *C. rastoni;* a full length cDNA encoding those toxins has been sequenced (Nagai et al., 2000a). CrTX-A was lethal to mice (20 μg/kg) and crayfish (5 μg/kg). Intradermal injection of CrTX-A in mice caused inflammation similar to one observed in victims stung by *C. rastonii.* Similar toxins, CaTX-A and CaTX-B, were isolated also from *C. alata* (Nagai et al., 2000b). Recently a neurotoxin with m.w. 120 kDa and three cytolysins with m.w. 36, 139 and 220 kDa were isolated from *C. marsupialis* (Sanchez-Rodriguez and Cruz-Vazquez, 2006), but no detailed pharmacological studies have been published.

4. Scyphozoa

4.1. *Cyanea capillata (Lion's mane jellyfish)*

The first report of on *C. capillata* stings described the pathophysiological effects of the venom on human skin (Kristenson, 1949). Later, it was shown that the venom liberates histamine from isolated rat mast cells (Uvnas, 1960). The venom contains a 70 kDa basic cardiotoxic protein that causes an irreversible contracture of skeletal muscles (Walker, 1977). Many of these effects might be due to an increase in intracellular calcium ion concentration, suggesting that at least one toxic component of the venom is a pore-forming toxin.

4.2. Chrysaora quinquecirrha *(Sea nettle)*

Species of *Chrysaora* are present in all oceans, but the toxicity of their venoms has not yet been determined. Contradictory results published in the literature may be due to different procedures of isolating the active constituents, as well as differences between species. Initially it was reported that the venom from the American *C. quinquecirrha* is lethal and that its principal action is on cardiovascular system (Burnett and Goldner, 1969). This finding was further supported by observation that the venom causes an irreversible contraction in the rat aorta which could be prevented either with Ca^{2+} channel blockers or by removal of Ca^{2+} from the bathing solution. It was concluded that the venom increases the influx of Ca^{2+} through Ca^{2+} channels (Lin et al., 1988). A later paper by the same laboratory found no histological changes in brain, heart or lungs, but a pronounced necrosis of liver and kidney (Muhvich et al., 1991). Reduction of blood flow to vital organs was observed only shortly before death of the experimental animals. Although the venom was toxic to cultured hepatocytes, its action was not Ca^{2+} dependent (Houck et al., 1996). Since the venom activates the complement system this might be, at least in part, responsible for the tissue damage (Ishikawa et al., 2004). A lethal factor of m.w. 105,000 Da was isolated from the venom (Long-Rowe and Burnett, 1994), but a pharmacological study of this constituent has not been reported.

At present it is clear that most toxic fractions isolated from different jellyfish venoms are comprised of a large number of proteins with a number of poorly defined pharmacological actions. Although antivenom against *C. fleckeri* venom reacts with some of the toxins from different jellyfish, it seems unable to prevent the harmful effects of those toxins which have been tested *in vivo* and *in vitro*. In spite of the great variety of proposed pharmacological actions of these fractions described in literature, it seems likely to this reviewer that cardiotoxicity is responsible both for human fatalities and for the lethal effects in experimental animals. Cardiovascular collapse may be due either to Ca^{2+} entry through newly formed pores, to the release of endogenous substances such as epinephrine or histamine, to interference with ionic channels in cell membranes, or a combination of these and other actions not yet identified. Toxin isolation, cDNA sequencing, and expression of recombinant toxins, especially from the most harmful jellyfish species, should enable a more detailed pharmacological analysis of the individual toxins and ultimately provide more rational basis for developing more effective treatments of envenomations.

5. Hydrozoa

5.1. Hydra *sp. (Hydra)*

Polypeptide toxins with cytotoxic, neurotoxic and pore-forming activities have been found in *Hydra*. Initial studies on the effects of substances released from nematocysts revealed a positive inotropic action in the vertebrate myocardium (Lesh-Laurie et al., 1989). The sustained increase in the amplitude and shortening of the rise-time of the force was similar to the effects described for substances from *Anthopleura* (Shibata et al., 1976). A detailed description of proteins isolated from hydra is described elsewhere in this volume (Sher and Zlotkin, in press).

5.2. Millepora *sp. (fire coral)*

Protein containing fractions from crude extracts of the Mexican stinging fire coral *Millepora complanata* elicited contractions of guinea-pig ileum when applied at concentrations from 0.001 to 1000 μg/ml. The contractions were reduced in Ca^{2+} free medium, and in the presence of nifedipine, an L-type Ca^{2+} channel inhibitor (Rojas et al., 2002). Later it was been shown that a crude extract obtained from this hydrozoan causes concentration-dependent contraction of isolated rat aortic rings. The effect was dependent on extracellular Ca^{2+} and was also diminished after depletion of intracellular Ca^{2+} stores. As the vasoconstrictor action of the crude extract did not depend on the presence of intact endothelium, a direct action on vascular smooth muscle was postulated. The extract also caused haemolysis, which might be due to a PLA2 activity, since haemolysis was reduced in the presence of PLA2 inhibitors (Ibarra-Alvarado et al., 2007). At present it is not known which of the components of the extract are responsible for

the above-mentioned effects, but the 15, 20 and 30 kDa m.w. polypeptides seem to be the most likely candidates.

5.3. *Physalia sp. (man o'war)*

The Man O'War *P. physalis* is actually belongs to the Class Hydrozoa, although it is considered a jellyfish by a majority of people. Its common name derives from the boat-like appearance of its float, which keeps it at the ocean surface and allows it to be distributed by air as well as water currents. It is composed of four types of specialized colonies of siphonophores. Its stings are extremely painful but rarely fatal. A severe envenomation by *P. physalis* was reported (Klein and Bradshaw, 1951). Initial studies of the pharmacological effects of *Physalia* venom were published a decade later, including effects on the cardiovascular system. *Physalia* toxin was one of the first cnidarian toxins to be isolated directly from nematocysts (Tamkun and Hessinger, 1981). Several proteins have been isolated from *P. physalis* venom. *Physalia* toxin (m.w.240 kDa) is composed of three heavily glycosylated subunits and is haemolytic and also lethal to mice when injected intraperitoneally. The mice died due to flaccid paralysis that was preceded by a period of hyperactivity. Later, it was shown that cardiovascular effects of the venom may be responsible for the lethality after i.v. injection in the rat and similar effects were observed on the canine cardiovascular system; the mechanism of its neurotoxic and cardiotoxic action is not yet known. However, the toxin causes conduction disturbances in the heart. Verapamil reduces the cardiotoxic action of the venom, but the utility of verapamil as a treatment for sting victims has not yet been demonstrated. Proteins from *P. physalis* nematocysts caused relaxation of the muscle vascular bed in dogs under pentobarbital anaesthesia, and it was concluded that prostaglandins were responsible for that effect (Loredo et al., 1985). Relaxation of vascular smooth muscle was confirmed on norepinephrine pre-contracted isolated rabbit arterial rings, and icreased endogenous prostaglandin synthesis was suggested as a mechanism of vasodilatatory action (Loredo et al., 1986). Vasodilation was also observed on a vascular bed that had been pre-treated with histamine.

Other authors have shown that venom from *P. physalis* releases histamine from mast cells by initially causing a rapid release of histamine by stimulating exocytosis, but ultimately causing lysis of mast cells (Cormier, 1984). Stimulation of exocytosis could be a result of Ca^{2+} entry into the cells, and indeed it has been shown that the nematocyst venom in a concentration dependent manner induces Ca^{2+} influx not only in cardiomyocytes but also in a number of other cell lines (Edwards and Hessinger, 2000; Edwards et al., 2000); some cell lines were more susceptible to the action of the whole venom. Calcium entry was accompanied by the release of lactate dehydrogenase, which could be at least partially prevented by addition of osmotic protectants to the medium. Ratiometric measurements of intracellular Ca^{2+} revealed that the venom increases $[Ca^{2+}]$ in the cells, and that the increase is preceded by a lag period that was increased only at very low concentrations (Edwards et al., 2002). The results imply that the venom contains pore-forming proteins (Edwards et al., 2002), but no direct evidence has yet been presented.

6. Final comments

While *in vitro* studies of toxin interactions with receptors can precisely reveal their basic mechanisms of action, they cannot alone always predict the action of the toxin or venom on an isolated organ or the whole organism. In the future, a greater communication between toxinologists working at different levels of biological organization is highly desirable, as it should facilitate the understanding and prediction of the whole animal effects of toxins, which can serve as a rational basis for the treatment of cnidarian envenomations.

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Conflict of interest

The author has no conflict of interest to declare.

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