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The phylum Cnidaria and investigations of its toxins and venoms until 1990

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ABSTRACT

Cnidarians are the largest phylum of generally toxic animals, yet their toxins and venoms have not received as much scientific attention as those of many terrestrial (snakes, scorpions, spiders, etc.) and even some marine animals (i.e. cone snails). Approximately 13,000 living cnidarian species have been described by systematists. A major rationale for their study in the past, besides scientific curiousity, was to better treat victims of their envenomation. While that goal remains a high priority, it is now appreciated that the toxins of these mostly marine animals can be very useful molecular probes for the analysis of ion channels involved in electrical signaling, immune responses and other signal transduction processes of biomedical interest. For instance, anaphylaxis was discovered by Richet (1905) during experiments with sea anemone and hydrozoan tentacular extracts. Similarly, it has recently been shown that a toxin from another sea anemone is able to potently inhibit T-lymphocyte proliferation in models of certain autoimmune diseases. Thus, these natural substances continue to be of relevance for understanding and treating human diseases. In addition to introducing phylum Cnidaria (Coelenterata), we provide a short history of early (until about 1990) research on cnidarian toxins and venoms, to provide a perspective for appreciating the scientific advances of the past two decades that are summarized in the ensuing 19 papers in this special *Toxicon* issue.

1. Phylum Cnidaria

Cnidarians are simply built animals with radial symmetry that contain only two layers of cells, ectoderm and endoderm. Mesoglea, a non-cellular matrix, is present between the two layers. These animals have also been referred to as coelenterates, as they possess a single body cavity, namely their digestive system (enteron). Cnidarians are primarily predators; after digesting their food, the undigestible materials are emitted from their oral opening back to the environment. Certain species may also scavenge dead animals or obtain nourishment from intracellular, photosynthetic unicellular algae, named zooxanthellae. Ctenophores (common name: "comb jellyfish") have the same body organization as cnidarians. They also have a similar digestive system and no other body cavity, and although some possess nematocysts, these are now known to be acquired from their cnidarian prey. Thus, most biologists today use the term Cnidaria rather than Coelenterata when referring to this phylum and put comb jellyfish in a distinct phylum. Cnidarians, like sponges and placozoans, are one of the oldest living animal groups. Since the oldest known cnidarian fossils, from the Precambrian period, are of polyp and medusoid forms, it is likely that the major cnidarian classes were already present at that time (Scrutton, 1979).

At least four toxic, living classes of cnidarians are currently recognized by most systematists. They are the

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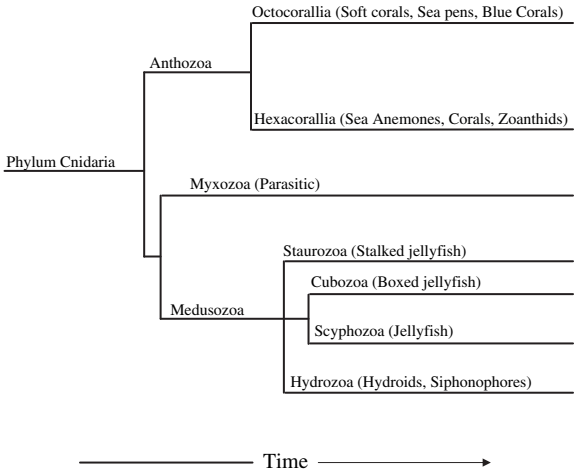

Fig. 1. Simplified phylogenetic "tree" of the phylum Cnidaria, based on a recent paper (Collins, in press). Only relationships of the various cnidarian classes are considered.

Anthozoa, Cubozoa, Scyphozoa and Hydrozoa. Representatives of the medusazoan (Fig. 1) branch of the phylum generally display both polyp and medusoid life stages, with the latter stage being responsible for sexual reproduction; free-living medusa are formidable predators on fish and other animals in all the oceans of the world, in no small part due to their armamentarium of poisonous nematocysts. In contrast, sea anemones, soft corals and sea pens (Anthozoa) are sessile polyps which can reproduce sexually as well as asexually. The only class of cnidarians that probably lacks a toxic venom is the Myxozoa, a group of approximately 2000 small parasitic animals that only recently were shown to belong to this phylum (Collins, in press). For a long time it was debated which class of cnidarians was basal in the evolution of the phylum. However, with the advent of molecular biological phylogenetic methodologies based on nucleic acid sequencing, it has been possible to conclude with confidence that the Anthozoa are the basal group in the cnidarian phylogenetic tree (Bridge et al., 1995; Collins et al., 2006). So the polyp likely preceded the medusoid form in the course of the phylum's evolution. It has been suggested that the two major groups of anthozoans, octacorals and hexacorals, deserve Class status (Collins, in press). Delineation of the phylogenetic relationships between the major classes in this ancient phylum is a remarkable achievement and will hopefully stimulate research on the biochemical evolution of the toxins found in these animals.

2. Cnidocytes: the cells that provide the venom

All cnidarians produces cnidae, now commonly referred to as cnidocysts. Nematocysts are cnidocysts that are used to capture prey, usually by injecting a venom; they are elaborated within the Golgi apparatus, and consist of a collagenous wall, an eversible, arrow-like delivery tubule (Fig. 2) and venom. While there are approximately 30 morphologically distinct types of cnidocysts, most cnidarians contain just a fraction of this number of possible types; the medusozoan classes display the greatest diversity of nematocyst types. The cells which contain these complex

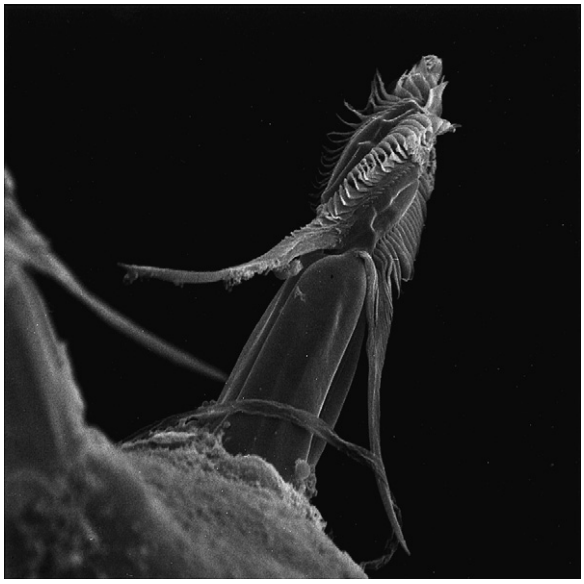

Fig. 2. Scanning electron micrograph of a large discharged stenotele tubule, capable of penetrating the chitinous cuticle of a small crustacean. The length of the tubule, bearing stylet appendages to anchor the most of the ejected tubule inside the prey, can exceed 30 μm (from Ostman et al., 1991).

capsules are referred to as cnidocytes or nematocytes; they are derived from stem cells referred to as cnidoblasts or nematoblasts. Cnidocytes make up a large fraction of the total number of cell types expressed in a given species. The cnidocyte surface possesses a sensory structure, the cnidocil, that is responsible for responding to prey or predator stimuli and triggering cnidocyst discharge. Cnidocytes are innervated and thus are in communication with the rest of the cnidarian organism, but they can also be individually stimulated to discharge, i.e., they are independent effectors. While only a small fraction (<1%) of the known cnidarian species have been implicated in human stings, it is likely that almost all non-parasitic members of the phylum are toxic to at least some animals. Already, toxins have been isolated from many species that are considered harmless on contact with human skin.

In the early years of cnidarian venom research the main focus was on the nematocysts, which were recognized as the venomous apparatus in sea anemones and jellyfish by several zoologists by the middle of the 19th century (Lenhoff and Lenhoff, 1988). The first detailed description of nematocysts was probably accomplished by Chun (1881). Almost a half century later, Weill (1930) described and classified the various types of nematocysts, based on their morphological features. Yanagita (1960) was the first to investigate the physiological mechanisms of nematocyst discharge. Then the Tardent group in Switzerland captured the rapid sequence of events associated with nematocyst discharge using high resolution time-lapse photographic methods (Holstein and Tardent, 1984; Tardent, 1995). Transmission and scanning electron microscopic techniques were used by Mariscal (1984) and others to reveal the fine structures of nematocysts. X-ray microanalysis allowed the precise measurement of concentrations of

particular cations (calcium, potassium, etc.) within undischarged nematocysts *in situ* (Tardent et al., 1990). The membrane signaling processes underlying cnidocyte response to stimuli and the associated nematocyst discharge were also investigated by electrophysiological recordings of the membrane events triggering exocytosis, using isolated cnidocytes (Anderson and McKay, 1987).

3. Investigations with toxic cnidarian extracts

In the first decades of the 20th century, it was practically impossible to isolate and chemically characterize toxins within venoms, as the biochemical techniques for isolating such natural products hardly existed, and no one had devised an efficient method for obtaining venom separate from the rest of the animal. However, the French physiologist Richet partially purified, studied and named two active components, "congestine" and "thalassine" from tentacular extracts of European sea anemones, primarily *Actinia equina* but also *Anemonia sulcata* (Richet, 1903a,b; Richet, 1905). Thalassine was subsequently found to liberate histamine from mast cells and, when injected into the skin, cause the release of a slow-reacting substance (SRS), that was later shown to be a prostaglandin (Jacques and Schachter, 1954). Although thalassine was initially thought to be the trimethylammonium ion (Ackermann et al., 1924), subsequent studies demonstrated that this simple amine lacks the actions of thalassine (Mathias et al., 1960). Hence, the identity of thalassine is still unknown. The congestine activity was likely due to equinatoxin, a pore-forming peptide (see below), as both produce pulmonary edema and cardiovascular effects and are found in the same anemone (Sket et al., 1974; also see Kristan et al., and Suput papers in this issue). Richet also reported the presence of a non-dialyzable toxin, "hypnotoxine," in tentacles of a hydrozoan siphonophore commonly referred to as the Portuguese man o'war. A large, complex protein later isolated from *Physalia* nematocysts may be the same as Richet's "hypnotoxine" (Tamkun and Hessinger, 1981). Richet later received the Nobel Prize for Physiology and Medicine, largely for his discovery of anaphylaxis while investigating the effects of toxic cnidarian extracts. Anaphylaxis is a potentially life-threatening immune hypersensitivity reaction to an antigen.

When aqueous extracts of the sea anemone *Adamsia palliata* were injected into shore crabs, they quickly developed convulsions, flexed their walking legs and frequently "autonomized" them, then became quiescent, flaccid and eventually died (Cosmovici, 1925). It was noticed that crustaceans, in comparison with other invertebrates, were especially sensitive to the *Adamsia* extract. An exception was the commensal (symbiotic) crab, *Eupagarus prideauxii*, which was unaffected by large doses of these extracts. It was demonstrated that the resistance of this crab to *Adamsia* toxins is due to an as yet unknown substance in its blood, which is absent in the other crustaceans that are sensitive to the toxins. Injection of a small amount of the haemolymph of *E. prideauxi* into a similar non-commensal species, the sensitive *Eupagarus bernhardus*, generated a temporary resistance to the *Adamsia* toxic extract (Cantacuzene, 1925).

4. Peptidic sodium channel toxins: the first type of cnidarian neurotoxin isolated

Although the Russian chemist Tswett in 1906 demonstrated the utility of column chromatography for the separation of some small molecules (carotenoids), it was another 50 years before this incredibly versatile method became widely used to isolate peptides and proteins from venoms. Indeed, examination of the proceedings of the first symposium on marine toxins revealed that even in the 1960s most investigators of marine toxins were still studying the effects of whole venoms or crude extracts (Nigrelli, 1960). While it is important to understand the actions of whole venoms, ultimately this can only be achieved after isolating and studying the individual constituents. Biological investigations of crude extracts are especially problematic, since active substances in tissues may be mixed with venom constituents. Thus, a new level of understanding of cnidarian toxins is made possible by their availability in homogeneous form. Here, the pioneering work of Laszlo Béress and his collaborators in Germany and France, who have purified and characterized several pharmacologically distinct neurotoxic peptides from European sea anemones over the past three decades, is especially noteworthy. From *A. sulcata* they initially isolated three peptide neurotoxins (Béress et al., 1975). The two larger, homologous peptides were soon shown to delay the inactivation process of voltage gated sodium channels (Rathmeyer and Beress, 1976). The amino acid sequence of *A. sulcata* toxin II was the first one to be reported for a cnidarian peptide (Wunderer et al., 1976). On the other side of the Atlantic Ocean, similar electrophysiological studies were carried out with a partially purified toxin fraction from the Caribbean sea anemone *Condylactis gigantea* (Shapiro, 1968; Narahashi et al., 1969). Like the scorpion α-toxins, these sea anemone toxins were shown to bind to so-called sodium channel site 3, located on the external surface of neuronal and cardiac membranes (Catterall and Beress, 1978).

About the same time, a group at the University of Hawaii was investigating the biochemistry and cardiac inotropic actions of similar peptides from two California sea anemones, *Anthopleura xanthogrammica* and *Anthopleura elegantissima* (Norton et al., 1976; Tanaka et al., 1977; Shibata et al., 1976). Extensive *in vivo* as well as *in vivo* studies were made on these peptides with the hope that they would be become useful therapeutic agents for treatment of congestive heart failure (Alsen et al., 1976; Renaud et al., 1986). Norton's group in Australia has pioneered in the NMR analysis of these and other peptide toxins; the tertiary structures of two type 1 neurotoxins, anthopleurin-A and *A. sulcata* Ia, were the first to be determined, using NMR methods (Norton and Norton, 1979; Gooley et al., 1984; Widmer et al., 1989; Norton, 1991)

Research groups in Marseilles (Schweitz et al., 1985), Vladivostok (Zykova et al., 1985) and Gainesville (Kem et al., 1989) later isolated homologous but distinct primarily neurotoxic peptides (so-called type 2 toxins) from another family of sea anemones, the Stichodactylidae, primarily found in Indopacific waters. A very high resolution NMR solution structure was obtained for one of these peptides,

ShI (Fogh et al., 1990). ShI was also the first cnidarian peptide toxin to be successfully synthesized and studied by solid-phase methods (Pennington et al., 1990a). A peptide toxin from a very different (acontiate) species of sea anemone, *Calliactis parasitica*, was homologous with the types 1 and 2 toxins, but its sequence was relatively unique (Cariello et al., 1989). Nevertheless, its action on arthropod sodium channels was later shown to be indistinguishable from those of the types 1 and 2 toxins (Salgado and Kem, 1992).

Once the sequences of these peptides were known, one of the next goals was to identify the sites on these molecules that are involved in binding to sodium channels. Initial studies (Barhanin et al., 1991; Gruen and Norton, 1985; Mahnir et al., 1989) relied upon "group-specific" reagents that had earlier been useful in covalently modifying particular kinds of amino acid side chains in proteins. These studies provided useful data that stimulated interest in defining the toxins' binding domains, but were limited by the poor group selectivity of the reagents and interpretational problems that resulted from multiple modifications occurring on the same peptide. Nevertheless, they allowed tentative identifications of important side chains, including some bearing carboxylate or guanidinyl groups. *De novo* chemical synthesis was successful in generating monosubstituted "mutants" of the type II toxin ShI (Pennington et al., 1990b). This study provided strong evidence that certain acidic side chains near the N-terminus of this type of toxin are important either for maintenance of the active folded structure, or are directly involved in the toxin's interaction with the sodium channel.

The smallest *A. sulcata* peptide toxin, As-III, contains only 27 residues and is thus much smaller than the ones discussed above (Béress et al., 1977; Martinez et al., 1977). Several other homologs were subsequently isolated and characterized (Ishikawa et al., 1979). These small sea anemone neurotoxins target crustacean, insect and possibly to a lesser extent, other animal sodium channels; however, no action on vertebrate channels has been reported to date. At least one of the toxins displays high affinity binding to crustacean nerves that is markedly dependent upon the membrane's electrical potential (Fujita et al., 1983).

While the scleractinian corals and related corallimorpharians, like sea anemones, are hexacorals (have six mesenteries rather than eight), their toxins have not been investigated nearly as intensively as those of the sea anemones. Most species occurring in Japanese and Australian coastal waters that were surveyed were toxic either to whole animals or to isolated cells (Hashimoto and Ashida, 1973; Gunthorpe and Cameron, 1990). However, so far the only scleractinian toxins that have been isolated and characterized are from corals of the genus *Goniopora*, whose polyps are readily harvested. First, a peptide toxin (9700 Da) was isolated from a *Goniopora* sp. occurring along the Japanese coast (Fujiwara et al., 1979). Apparently the sequence of this first purified coral toxin was determined; it was reported to contain 88 amino acid residues, including 10 half-cystines (Ashida et al., 1987). Electrophysiological studies revealed a mechanism of action similar to that of the sea anemone neurotoxins, namely delaying and inhibiting sodium channel inactivation (Muramatsu et al., 1985; Gonoi et al., 1986). Also, a calcium channel blocking protein (molecular size 19,000 Da) was purified from a *Goniopora* species collected along the Jordanian coast of the Red Sea (Qar et al., 1986).

5. Cytolytic proteins

Besides the neurotoxins, cytolytic sea anemone toxins attracted considerable interest starting in the 1970s. Hessinger and Lenhoff (1973) discovered an interesting cytolytic system in acontial nematocyst venom of the sea anemone *Aiptasia pallida*. They were the first to report the existence of a phospholipase A in a cnidarian venom. Together with a proteinaceous "indirect lytic factor," *Aiptasia* venom was hemolytic. The first representative of the large, so-called "actinoporin" (Kem, 1988; Turk, 1991) group of cytolysins to be isolated was equinatoxin, named after *A. equina*, its source (Ferlan and Lebez, 1974). Later, it was shown that equinatoxin is actually a mixture of three isotoxins of which equinatoxin II is the most abundant one (Maček and Lebez, 1988). Devlin reported the occurrence of a potent hemolytic protein in the Caribbean sun anemone, *Stoichactis* (now *Stichodactyla*) *helianthus* (Devlin, 1974). This hemolysin was further studied in some detail by Bernheimer and Avigad (1976). They showed that the active peptide fraction, later shown to contain two major isotoxins (Kem and Dunn, 1988), displayed a molecular size of approximately 20 kDa by SDS gel electrophoresis and was very basic. They also discovered the sphingomyelin affinity of this toxin and demonstrated that the presence of this lipid was almost indispensable for its cytolytic permeabilization of living cells and liposomes. Soon it was shown that the toxin forms ion channels in artificial membranes (Michaels, 1979). The first reported "actinoporin" sequence was for *Stoichactis* cytolysin III (Blumenthal and Kem, 1983). Unfortunately, an important hydrophobic peptide fragment from near the N-terminus was lost during HPLC purification of the various peptide fragments. The missing part of the sequence was later accounted for by mass spectrometric analysis, showing that *Stoichactis* cytolysin III is the same as sticholysin II (Stevens et al., 2002). The crystal structures of two actinoporins, sticholysin II and equinatoxin II, and the solution structure of tenebrosin-C have been published in recent years and will be discussed in several papers appearing in this issue.

Almost 40 sea anemone species have been screened for the presence of hemolytic proteins so far. Most of these proteins share common characteristics with equinatoxins and sticholysins (see chapters in this issue). A notable exception was the a cholesterol-inhibitable protein, metridiolysin, discovered by Bernheimer and Avigad (1978).

Scyphozoan, cubozoan and hydrozoan venoms often display cytolytic (sometimes including hemolytic) and/or membrane depolarizing properties (Keen, 1969; Kleinhaus et al., 1973; Burnett and Calton, 1977; Walker, 1977; Kihara et al., 1988). However, their isolation, particularly from crude tentacular extracts, was found to be extremely difficult, and this has retarded their scientific investigation. However, density gradient centrifugation methods for the isolation of at least small amounts of different nematocysts

have been available for some time (Endean and Rifkin, 1975; Kem and Östman, 1991).

6. Non-peptidic toxins

Besides peptide and protein toxins, some very interesting non-peptidic toxins have been identified in certain anthozoans. These include a huge group of cyclic diterpenes that occur in the octocoral anthozoan branch of the phylum; their diverse structural diversity (>500 compounds have been identified to date) was recently surveyed (Berrue and Kerr, 2009). Apparently the first such compound to be identified was sarcophine (Neeman et al., 1974). One of the most pharmacologically interesting diterpenes is lophotoxin, isolated from a Pacific gorgonian (Fenical et al., 1981). This cyclic compound irreversibly blocks neuromuscular nicotinic acetylcholine receptors by forming a covalent bond between one of its two epoxide groups and an important tyrosyl side chain in the ACh binding site (Abramson et al., 1988). Other types of compounds that have been found in octacorals include prostaglandins (Weinheimer and Spraggins, 1969), the muricin saponins (Bandurraga and Fenical, 1985) and pseudopterosins (Look et al., 1986); the last two groups contain carbohydrate moieties.

The most potent marine toxin, palytoxin, is a complex polyether compound that was originally isolated from zoanthids (Anthozoa) of the genus *Palythoa* (Moore and Scheuer, 1971). Determining the structure of this toxin was very challenging in those times before more powerful NMR pulse protocols became available (Moore and Bartolini, 1981; Uemura et al., 1981). Armstrong et al. (1989) managed to synthesize a protected palytoxin carboxylic acid. Later refinement of this elaborative synthesis led to palytoxin identical with the natural compound, but required no less than 65 steps (Kishi, 1989; Su and Kishi, 1994). The unique mechanism of action of this toxin will be described later in this issue.

7. Treatment of cnidarian envenomations

Jellyfish can be a serious hazard for public health due to their painful sting and possible fatal outcome. Earliest reports of stings mainly concerned *Physalia* sp. stings (Lowry, 1911; Russell, 1966). Treatment of such a sting by alcohol was reported almost simultaneously by Bullard (1911). The deadliest marine cnidarian, the cubozoan *Chironex fleckeri*, inhabits the coastal waters of northern Australia. Pioneering work on this deadly animal and its venom was done by Flecker, just after World War II. Research on jellyfish stings intensified in the 70s after several fatal cases of stings inflicted on bathers by Australian cubozoans were reported (Flecker, 1952; Southcott, 1958; Barnes, 1964; Baxter and Marr, 1969).

From the venom and nematocysts of *C. fleckeri*, Crone, Endean and others tried to isolate the proteins responsible for the toxic manifestations of this life-threatening envenomation. Similarly, Burnett and Calton (summarized in their 1977 review in Toxicon) partially purified toxins from the sea nettle, *Chrysaora quinquecirrha*, which stings bathers in Chesapeake Bay, USA. Denaturing (SDS) electrophoresis was used to examine the various size classes of proteins in the venoms, but this was before techniques for removing the resolved proteins from the gel and sequencing them had been introduced. A very large hemolytic protein (~260,000 Da) was also isolated from nematocysts of a Mediterranean jellyfish, *Rhizostoma pulmo* (Cariello et al., 1988). Although these early attempts at purification and biochemical characterization were only partially successful, they provided at least a general picture of the complexity of cubozoan and scyphozoan venoms (Crone, 1971; Endean and Noble, 1971; Burnett and Calton, 1977).

8. Concluding comment

Our short historical account of cnidarian toxinological investigations ends in 1990, shortly after the proceedings of a symposium that focused on nematocyst biology was published (Hessinger and Lenhoff, 1988). The current issue of *Toxicon* is apparently the first collection of papers that focuses on cnidarian toxins and venoms.

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