FRACTIONATION, PRIMARY STRUCTURAL CHARACTERISATION AND BIOLOGICAL ACTIVITIES OF POLYPEPTIDES FROM THE VENOM OF THE PLATYPUS (*ORNITHORHYNCHUS ANATINUS*)

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G. M. de Plater BSc. (ANU)

The John Curtin School of Medical Research
Australian National University
Canberra

STATEMENT

All the work described in this thesis is original and represents work carried out by myself, except where specifically acknowledged, under the supervision of Dr Rosemary Martin and Dr Peter Milburn, from October 1994 to June 1998. During this time a number of presentations were made at scientific meetings. The following abstracts were published in conjunction with these presentations.

de Plater, G. M., Martin, R. L. and Milburn, P. J. (1996) The Natriuretic peptide (ONP-39) from platypus (*Ornithorhynchus anatinus*) venom promotes mast cell histamine release. Platypus Biology - A National Symposium.

de Plater, G. M., Martin, R. L. and Milburn, P. J. (1998) Platypus (*Ornithorhynchus anatinus*) venom activates a Ca^{2+} -dependent inward current in rat dorsal root ganglion neurones. *Proc. Aust. Neurosci.* **9**: 72

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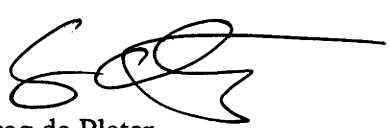
The following papers, which have been accepted for publication, describe some of the work presented in this thesis:

de Plater, G. M., Martin, R. L. and Milburn, P. J. (1998) The natriuretic peptide (ovCNP-39) from platypus (*Ornithorhynchus anatinus*) venom relaxes the isolated rat uterus and promotes oedema and mast cell histamine release. *Toxicon* **36:** 847-857

de Plater, G. M., Martin, R. L. and Milburn, P. J. (1998) A C-type natriuretic peptide from the venom of the platypus (*Ornithorhynchus anatinus*): structure and pharmacology. *Comp. Biochem. Physiol. C. In press.*



Greg de Plater

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ABSTRACT

Platypus, *Ornithorhynchus anatinus*, envenomation produces marked short- and long-term *sequelae*, consisting of intense pain, oedema and lasting hyperalgesia. This thesis describes the characterisation of polypeptides and biological activities from *O. anatinus* venom (*Oa*V) which may contribute to these effects.

SDS-polyacrylamide gel electrophoresis was initially used to compare *Oa*V samples taken from excised crural glands and from live animals at different times of year. Most protein components were present in all samples, although the relative proportion of each component varied markedly, particularly between samples taken during and outside the breeding season. Several of the major *Oa*V polypeptides were purified using high-performance liquid chromatography (HPLC) and subjected to N-terminal Edman sequencing. Low levels of similarity between these and other proteins contained in sequence databases was observed with the exception of a C-type natriuretic peptide (ovCNP-39), nerve growth factor (NGF), a Kunitz-type protease inhibitor-like protein and two likely members of the lipocalin superfamily.

The purification, detailed structure, synthesis and pharmacological characteristics of ovCNP-39 (*Ornithorhynchus* venom C-type natriuretic 39-residue peptide) are described in this study. The peptide was found to share a high degree of homology with eutherian CNPs. These peptides produce hypotension *in vivo* and relax smooth muscle *in vitro* but are poorly characterised in terms of physiological function. ovCNP-39 was equipotent with human/rat/porcine CNP-22 in eliciting cyclic guanosine 5'-monophosphate (cGMP) elevation in cultured vascular smooth muscle cells. This

suggests that, like CNP-22, it acts through the ANP_B natriuretic peptide receptor subtype. The direct elevation of cGMP in vascular smooth muscle by ovCNP-39 may underlie the vasodilatory effects of platypus envenomation. The possibility that ovCNP-39 contributes to the acute effects of envenomation was also investigated and it was found that injection of ovCNP-39 or eutherian CNP-22 into the rat hind-paw produces oedema; both peptides also induced release of histamine from rat peritoneal mast cells. Two synthetic peptides, ovCNP-39(1-17) and ovCNP-39(18-39), corresponding to the N- and C- termini respectively, were found to be equipotent histamine releasers, suggesting that ovCNP-39 and, by analogy, other natriuretic peptides do not act through conventional natriuretic peptide receptors on mast cells.

To address the possibility that *Oa*V has a direct effect on nociceptive neurones, it was transiently applied (10 s) to small-medium (20-40 μm) diameter, cultured rat dorsal root ganglion (DRG) neurones. These cells have been shown previously to contain a proportion of neurones which express nociceptor properties, such as sensitivity to algogenic mediators and substance P-like immunoreactivity. In DRG neurones voltage-clamped at -60 mV, a 10 s application of *Oa*V at pH 7.4 had little or no effect. At pH 6.1, the venom produced a large inward current in 72 % of neurones, which was characterised by multiple transient events and, on average, lasted for several minutes. The Ca^{2+}-ATPase inhibitor, thapsgargin (1 μM) significantly reduced the current, suggesting its activation was dependent on the release of intracellular Ca^{2+}. All *Oa*V HPLC fractions which induced an inward current in DRG neurones contained a 12 kDa protein. This protein was shown to have a high degree of homology with NGF. An inhibitor of the TrkA NGF receptor, k-252a (200 nM) significantly reduced the inward current produced by whole *Oa*V. However, purified mouse β-NGF did not elicit inward currents, raising the possibility of a synergy between *Oa*V NGF and another as yet unidentified *Oa*V component.

Of the 94 residues of amino acid sequence obtained from *Oa*V NGF, 90 % and 67 % identity was observed with mouse β-NGF and Cobra (*Naja naja atra*) venom NGF, respectively. The strong structural similarity with β-NGF (and Type I snake venom NGFs) was supported by the comparable molecular weight of *Oa*V NGF which was found to be 13,328.

The experiments described in this thesis have implicated a number of *Oa*V polypeptides in the pathology associated with *O. anatinus* envenomation. Several novel and interesting findings have been made. In particular, no C-type natriuretic peptides have hitherto been purified and sequenced from an animal venom and, although NGF is found in many snake venoms, its presence in *Oa*V is highly interesting given the remarkable similarity between the long-term *sequelae* resulting from NGF administration and *O. anatinus* envenomation in humans. In addition, evidence has been provided for the first time that a venom may contain factors which act directly on nociceptive neurones.

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ABBREVIATIONS

ACE	angiotensin-converting enzyme
ASIC	acid-sensitive ion channel
AIEX	anion-exchange
ANGIS	Australian National Genome Information Service
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BCM	Baylor College of Medicine
BDNF	brain-derived neurotrophic factor
BEAUTY	BLAST Enhanced Alignment Utility
BLAST	Basic Local Alignment Search Tool
BNP	brain natriuretic peptide
BPTI	bovine plasma trypsin inhibitor
BSA	bovine serum albumin
CCTI	cow colustrum trypsin inhibitor
CFA	complete Freund's adjuvant
CGS	crural gland secretion
cGMP	cyclic guanosine 3', 5'- monophosphate
CGRP	calcitonin gene-related peptide
CICR	calcium-induced calcium release
CIEX	cation-exchange
CNP	C-type natriuretic peptide
DAG	diacylglycerol
DES	diethylstilboestrol
diHETE	dihydroxyeicosatetraenoic acid
DMEM	Dulbecco's modified Eagle's medium
DNP	*Dendroaspis* natriuretic peptide
DRG	dorsal root ganglion
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)
eNAP	equine neutrophil antimicrobial peptide
EST	expressed sequence tag

FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FMOC	fluorenylmethyl chloroformate
GDILP	GDP-dissociation inhibitor-like protein
GDP	guanosine diphosphate
GLP	granulin-like peptide
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
HP-GPC	high-performance gel permeation chromatography
HPLC	high-performance liquid chromatography
5-HT	5-hydroxytryptamine
IGFBP	insulin-like growth factor binding protein
IL	interleukin
IP_3	inositol-1,4,5-triphosphate
KLP	Kunitz-type protease inhibitor-like protein
LTB_4	leukotriene B_4
LTD_4	leukotriene D_4
MES	2-[N-morpholino]ethanesulfonic acid
M_r	relative molecular mass
NGF	nerve growth factor
NMDA	N-methyl-*D*-aspartate
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
*Oa*V	*Ornithorhynchus anatinus* venom
OPA	*ortho*-pthalaldehyde
ovCNP	*Ornithorhynchus* venom C-type natriuretic peptide
OVL	*Ornithorhynchus* venom lipocalin
PAF	Platelet-activating factor
PAS	periodic acid-Schiffs reagent
PBA	phosphate-buffered saline with 0.1% BSA
PBS	phosphate-buffered saline
PG	prostaglandin
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PKC	protein kinase C
PVDF	polyvinylidene difluoride

RP-HPLC	reversed phase high-performance liquid chromatography
RYR	ryanodine receptor
SCR	sequence conserved region
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
TEA	tetraethylammonium chloride
TFA	trifluoroacetic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
TOF-MALDI	time-of-flight matrix-assisted laser desorption/ionisation
Tris	Tris[hydroxymethyl]aminomethane
TTX	tetrodotoxin
TTX-R	tetrodotoxin-resistant
TTX-S	tetrodotoxin-sensitive
VR	vallinoid receptor

CHAPTER 1

GENERAL INTRODUCTION

The male platypus *(Ornithorhynchus anatinus)* bears a canalised, keratinous spur on each hind-limb which is attached, *via* a distensible duct, to a crural venom gland. Although it is clear that spurring produces excruciating, intractable pain and oedema in humans, very little is known about how *O. anatinus* venom (*Oa*V) produces these effects. It was the aim of the work presented herein to identify and characterise the constituents of *Oa*V which might be important contributors. Ultimately, piecing together how these constituents contribute to the overall envenomation pathology will facilitate our understanding of how *Oa*V works. This has implications not only for our understanding of the role of *Oa*V in the ecology of *O. anatinus* but also for our understanding of the processes underlying long-lasting inflammatory pain.

The following sections discuss the effects of *Oa*V in humans and experimental animals, and detail previous laboratory investigations into its composition and biological activities. As pro-inflammatory effects, particularly prolonged pain, comprise the major symptoms of envenomation, current knowledge on pain-producing inflammatory mediators and toxins has been reviewed. In the context of this, possible modes of action of *Oa*V and how these may be addressed experimentally, are discussed.

Venoms and natural toxins - nomenclature and general features

A large number of terrestrial and marine organisms produce "toxins", which may be defined as substances of plant, animal, or bacterial origin that are both foreign and adversely affect the target organism (Vogt, 1970). According to this definition, the

term also depends on the relation of the substance to the victim. For example, digitalis, a cardioactive alkaloid from the foxglove (*Digitalis purpurea*), which may be fatal following accidental ingestion, would not be considered a toxin when used in a therapeutic context.

The term "venom" is commonly used to describe the complete secretion of a toxin-producing gland associated with specialised wounding apparatus (Temple-Smith, 1973; Chahl and Kirk, 1975). Thus toxin-secreting fish and amphibians which lack such an apparatus are not considered "venomous". While a venom may be considered a toxin, the term "toxin" is generally restricted to purified venom fractions which satisfy the definition given above.

The use of venoms both for food procurement and self-protection has arisen convergently over a range of phyla. Venoms appear to have a primary but not exclusive role in either capacity. For example, snake venoms contain neurotoxins and enzymes which respectively facilitate the immobilisation and digestion of prey items, suggesting a primary role in predation. However, the incidence of snake envenomation in humans is testimony to the fact that the venom is also used defensively.

The venomous characteristics of *Ornithorhynchus anatinus*

The platypus (*Ornithorhynchus anatinus*) and its venomous characteristics in particular, have experienced a long history of being treated with disbelief or ignored completely. For example, the first specimen of *O. anatinus* sent to Britain in 1798 was thought to be a fake, stitched together by a taxidermist using mammalian parts and a duck bill (Grant, 1995). A recent review by Dufton (1992), entitled "Venomous Mammals", made no mention of *O. anatinus* and a recent case report of *O. anatinus* envenomation (Tonkin and Negrine, 1994) in the British Journal of Hand Surgery was accompanied by the following, apparently facetious, Editor's note: "A spate of Dodo

bites has recently been recorded in a remote area of New South Wales, and will be reported by Dr Tonkin in the next issue of the Journal". This is despite the fact that several publications exist which deal specifically with the subject, most notably, Martin and Tidswell (1895), Kellaway and LeMessurier (1935), Temple-Smith (1973) and Fenner *et al.* (1992).

O. anatinus is a rather unusual, oviparous mammal, which is indigenous to the river systems of eastern Australia. It has a soft bill which superficially resembles that of a duck; webbed feet; and a single tract for feeding, reproduction and excretion, which places it in the order Monotremata (Grant, 1995). However it possesses the following distinctly mammalian characteristics: it has hair, lactates and thermoregulates (although maintaining a relatively low body temperature of 32°C; Grant, 1995).

O. anatinus and three species of insectivore (*Blarina brevicauda*, *Solenodon paradoxus* and *Neomys fodiens;* see Dufton, 1992) comprise the only extant venomous mammals. Although the echidnas (*Tachyglossus setous* and *Tachyglossus aculeatus*) possess venom apparatus similar to that of the platypus, there is no evidence that it is functional (Calaby, 1968).

Structure of the venom apparatus

The venom apparatus of *O. anatinus* is restricted to the male and externally consists of a pair of movable calcaneus spurs (*cornu calcari*) on each hind-limb. Each spur normally lies against the limb but is attached at its base to an articulating bone (*os calcaris*), which allows it to be erected at right angles to the limb when required (Lewis, 1963; Temple-Smith, 1973). At the back of the tarsus is a sac or reservoir from which a tiny duct extends through the center of the spur (Martin and Tidswell, 1894; Lewis, 1963; Temple-Smith, 1973). A large distensible duct, which superficially traverses the biceps muscle, connects the reservoir to the crural venom gland. The

structure of the crural gland was originally described in detail by Martin and Tidswell (1894) who noted that it is divided into lobules containing numerous alveoli which are lined by a single layer of columnar epithelial cells on a basement membrane. During the breeding season, these cells attain their maximum size and extrude secretory vesicles. This period is therefore referred to as the extrusion phase. The secretory epithelium then undergoes sequential phases of regression, quiescence and elaboration before the next breeding season (Temple-Smith, 1973). These histological observations suggest a temporal relationship between breeding status and crural gland secretory activity (Temple-Smith, 1973).

Effects of envenomation

Although the clinical manifestations of *O. anatinus* envenomation have been systematically investigated in only two cases (Fenner *et al.*, 1992; Tonkin and Negrine, 1994), many anecdotal accounts exist and provide a useful source of detailed and consistent information on post-envenomation *sequelae.*

The earliest documented case of envenomation appears to be that of Jamieson (1817, cited in Martin and Tidswell, 1895), who, in a communication to the Philomatic Society of Paris, described the effects of envenomation: "...[the platypus] stuck its spurs into the palm and back of his right hand with such force, and retained them in with such strength, that they could not be withdrawn until it was killed. The hand instantly swelled to a prodigious bulk...The pain from the first was insupportable...[he] did not recover the perfect use of his hand for nine weeks". The extensive oedema and lasting pain described by Jamieson are consistently reported in most of the subsequent anecdotal accounts. For example, Spicer (1876) cites a case where "...The pain was intense and almost paralysing. But for the administration of small doses of brandy, he would have fainted on the spot: as it was, it was half an hour before he could stand

without support: by that time the arm was swollen to the shoulder, and quite useless, and the pain in the hand very severe...". Martin and Tidswell (1894) report a similar case: "...Mr. E. all the time suffered intense pain, and presently the wounded finger, then the hand, and ultimately the whole arm up to the shoulder swelled to a serious extent...".

Temple-Smith (1973) confirmed these anecdotal reports of the painful effects of *O. anatinus* venom by injecting a small quantity into his forearm. This resulted in "immediate intense pain which diminished to a dull muscular pain, impairing use of the arm for 48 hours".

Fenner *et al.* (1992) provided the first clinical case report of *O. anatinus* envenomation in a human subject. They report that the affected patient, who received spur wounds to the hand, presented with oedema and lasting severe pain which did not respond effectively to morphine and was only alleviated following a wrist block. Laboratory blood tests revealed an increased erythrocyte sedimentation rate (ESR), indicating possible coagualopathy. The pain in this case persisted for several months and substantially impaired use of the affected limb. Intense pain and oedema were similarly described in a more recent case reported by Tonkin and Negrine (1994), although the symptoms in this case subsided within several weeks.

From the anecdotal accounts and the clinical case studies, it is apparent that pain and oedema are the major symptoms associated with platypus envenomation. There does not appear to be any evidence of systemic neurotoxicity, myotoxicity[1], or necrotic effects and there have been no reported human fatalities.

[1]It should be noted that Fenner *et al.* (1992) reported "significant" forearm muscle wasting in the affected patient two weeks after envenomation but it was not established whether this was due to disuse of the affected limb due to the severe pain or caused directly by the venom.

Putative functions

Five major views regarding the function of the venom apparatus of *O. anatinus* have been suggested: (a) that it serves a role during copulation (Home, 1802; Burrell, 1927), (b) that it is used for food procurement (Fleay, 1956 cited in Calaby, 1968), (c) that it is merely a vestige, *i.e.* a remnant of an era when conditions were very different from those under which the animal now exists (Nicols cited in Martin and Tidswell, 1895), (d) that it is used for "toilet purposes" (Baden-Powell cited in Tidswell, 1906), and (e) that it serves as a weapon of offence and/or defence (Wood-Jones, 1923).

Home (1802) in his original description of the platypus suggested the spurs were used during copulation to prevent the female from escaping. Burrell (1927) also inclined to this belief, largely on the basis of the restriction of venom apparatus to the male, the "convenient" placement for such a purpose and the alleged presence of corresponding hairless depressions in the female. However, as Martin and Tidswell (1895) point out, for the male to apply its spurs to these depressions "would involve an amount of gymnastic ability of which even an *Ornithorhynchus* is incapable". In addition, Temple-Smith (1973) noted the relative absence of spur wounds on females compared with males, further increasing the scepticism for Home's hypothesis.

Fleay (1956, cited in Calaby, 1968) observed a platypus using its spurs on a frog which it eventually ate and subsequently suggested that the venom may be used to immobilise prey items. However, platypuses largely feed on annelids, insect larvae and small crustaceans and have a specialised anatomy for this type of procurement (Grant, 1995). The fact that the venom apparatus is restricted to the male also suggests that this is an unlikely function.

The naturalist, Nicols (cited in Martin and Tidswell, 1895), believed the apparatus to be a vestigial remnant of conditions very different from those under which the animal now exists. This and similar suggestions (*e.g.* Benett cited in Martin and

Tidswell, 1895) were based largely on observations that the animals were rather docile and made no attempt to use their spurs during handling. However, several cases of envenomation have occurred during the handling of injured animals (Spicer, 1876; Martin and Tidswell, 1895) and hunting dogs have been envenomed while attempting to retrieve wounded platypuses (Burrell, 1927). This suggests that *O. anatinus* has to be sufficiently threatened before it will deploy its spurs.

Baden-Powell suggested that the apparatus may be used for "toilet purposes" (cited in Tidswell, 1906). Again this appears unlikely because the apparatus is confined to the male, the spurs are located in an unsuitable position (Tidswell, 1906), and there are no reported observations of the animal using its spurs in this manner. In addition, the spur is canalised and attached to an exocrine gland suggesting a more specialised role.

The prevailing view is that the venom apparatus serves as "an offensive and defensive weapon" (Wood Jones, 1923). It is offensive in that it appears to be involved in resolving intraspecific, competitive interactions between males. This is supported by the following evidence: only males possess venom apparatus; secretory activity is temporally related to breeding status; a higher incidence of aggressive encounters occurs between males; and males harbour more spur wounds (Temple-Smith, 1973). Temple-Smith (1973) describes one particularly striking example of an aggressive encounter between two males: "At Bredbo during the breeding season, a large adult male which had shown no aggression towards three adult females held in the same container, fought viciously with a second male caught later that night and caged with them. The two males spurred each other frequently, gripping each other so tenaciously that it was impossible to separate them."

Where viable in terms of defendability, males tend to occupy mutually exclusive home ranges, each of which may contain several females (Serena, 1994; Gardner and

Serena, 1995). Where their home ranges overlap, they largely avoid each other (Gardner and Serena, 1995). The venom apparatus may play an important role in maintaining these behavioural patterns and, as a consequence, facilitate the monopolisation of breeding females by dominant males.

The accounts of envenomation in humans outlined in the previous section suggest that the venom also serves a protective function. In several such cases, the animal had been injured and subsequent handling apparently induced it to use its spurs in defence.

Composition and physiological effects of the venom

There has been an apparent reluctance to refer to the material secreted from the crural gland as "venom". It has been ubiquitously referred to as crural gland secretion (CGS) or crural gland extract, depending on the method of preparation (Martin and Tidswell, 1895; Temple-Smith, 1973). Perhaps this reflects uncertainty over the function of the crural system. However, the crural gland is clearly associated with a wounding apparatus and the secretion does produce adverse effects in humans and experimental animals (Martin and Tidswell, 1895; Kellaway and LeMessurier, 1935; Temple-Smith, 1973). It therefore conforms to the aforementioned definition of a venom and will be referred to as such in this thesis.

The earliest examination of the composition and physicochemical properties of the venom was conducted by Martin and Tidswell (1895). They expressed both "limpid"[2] and "opalescent" secretion from dissected crural glands, and precipitated it using 92% alcohol. The precipitate was mostly soluble in water, forming an "opalescent" liquid. It was shown to contain at least two "proteids", one, an "albumen", precipitated by heat and one, a "proteose", soluble at 100 °C. The secretion

[2] Limpid = Pellucid, clear, not turbid.

was injected subcutaneously into a rabbit and resulted in localised swelling and tenderness which subsided over a couple of days. When three rabbits were injected intravenously, a rapid fall in blood pressure, respiratory distress, including "expiratory convulsions", and death followed. Post-mortem examination revealed that two animals probably died from extensive intravascular coagulation. However, blood from the remaining animal, in which the material was injected more slowly (and which died somewhat later than the others), exhibited no signs of clotting in vessels. In fact a sample of this animal's blood was found to clot abnormally slowly. Martin and Tidswell concluded that the effects of the venom on blood pressure, coagulation and tissue oedema were analogous to those produced by Australian snake venoms. Noc (1904, cited in Calaby, 1968) found that the material (supplied by Martin) coagulated plasma which had had its clotting activity reduced by decalcification with citrate, oxalate and other reagents, and this property was destroyed by heating at 80 °C.

Kellaway and LeMessurier (1935) extended the initial observations of Martin and Tidswell using material supplied by Martin which was "...probably 30 years old". This was injected intravenously into two rabbits and resulted in severe dyspnoea[3] lasting for 7 or 8 minutes, but the animals completely recovered within 13 minutes. Some delay was observed in the clotting time of blood samples taken from these animals. However, the material promoted the coagulation of guinea pig citrated plasma *in vitro*. When injected subcutaneously, the material produced an extensive haemorrhagic oedema. Using freshly obtained material, Kellaway and LeMessurier demonstrated it to be feebly haemolytic but found that it produced a rapid and profound fall in blood pressure and death when intravenously injected into anaesthetised rabbits. The fall in blood pressure appeared to be associated with peripheral vasodilatation and was

[3] dysponoea - difficult or laboured breathing

observed following intravenous injection in a decerebrate cat, suggesting that it was not dependent on the use of anaesthetics. In one injected rabbit, particles of "whipped-out fibrin" were found in the heart upon post-mortem examination, confirming the pro-coagulant activity observed by Martin and Tidswell (1895). However, the fall in blood pressure was not dependent on intravascular coagulation (Kellaway and LeMessurier, 1935).

Kellaway and LeMessurier further tested the material using several *in vitro* preparations. They found that the venom produced vasodilatation in the perfused rabbit ear and in cat mesentery, supporting the notion that the fall in blood pressure was peripheral in origin. The venom also contracted the isolated guinea pig uterus and rabbit jejunum.

The most recent and comprehensive work on *O. anatinus* venom was that by Temple-Smith (1973). This study investigated the composition and physiological effects of the venom as well as the seasonal dependence of its secretion. Using gel electrophoresis, Temple-Smith demonstrated that the venom contains from 7 to 10 proteins, depending on the sample analysed. These were mainly "prealbumins" (Mr < 70 kDa) and "postalbumins" (Mr = 70-80 kDa) although the material also contained 1 - 5 larger proteins (Mr > 90 kDa). There was some seasonal variation in the relative expression of these proteins, with "postalbumin" proteins predominating in material taken from extrusion-phase glands but being absent or present in decreased concentrations in quiescent glands. The "postalbumin" components were probably mucoproteins as they were strongly PAS (periodic acid-Schiff's reagent)-positive, indicating the presence of carbohydrate. When the venom was injected subcutaneously into mice, some individuals squealed and were observed to lick, scratch and bite the injection site. These behavioural symptoms are typical indicators of pain and are

sometimes used quantitatively in behavioural studies of nociception (Bjorkman *et al.*, 1994; Narita *et al.*, 1996).

In Temple-Smith's (1973) investigation, the venom was fractionated by gel permeation chromatography and the fractions assayed for both lethality (following i.v. injection) in mice and "cutaneous activity" (plasma extravasation). Lethality was associated with high-molecular weight material which eluted in the first and apparently most concentrated (as judged by absorbance at 280 nm) fraction. This fraction and a lower molecular weight fraction also produced plasma extravasation in the skin of the rabbit. Lyophilisation of the venom dramatically reduced both of these activities and resulted in the loss of high molecular weight components. Temple-Smith (1973) therefore proposed that the lethal fraction was a high molecular weight component. This fraction was turbid and contained what was believed to be membrane and cell debris. However, this interpretation is equivocal as neat venom did not appear to be turbid and only became so upon dilution, which "reduced the viscosity of the secretion so that suspended material was precipitated" (Temple-Smith, 1973). It was perhaps more likely due to protein aggregation which may have been exacerbated by the low ionic strength of the diluent (distilled water was used). This notion is supported by the observation that gel electrophoresis of the lethal fraction failed to resolve any proteins apart from one band of "proteinaceous material" at the origin of the gel (Temple-Smith, 1973).

Temple-Smith (1973) found that mice which had received lethal or sub-lethal doses of venom typically exhibited hyperventilation, convulsions, cyanosis and apparent hind-limb paralysis. Post-mortem examination revealed no tissue or vascular damage except in the lungs where vascular damage, constricted alveoli and oedematous interalveolar septae were evident. In addition, large accumulations of erythrocytes were observed in alveolar capillaries, suggesting that the flow of blood through the

lungs had been reduced by obstruction or constriction of the pulmonary arteries. Thus, the behavioural and post-mortem observations of Temple-Smith suggested that death resulted from respiratory failure, although probably indirectly given that the venom lacks curare-like activity (Kellaway and LeMessurier, 1935; Temple-Smith, 1973). Blood samples from animals receiving lethal doses of venom took longer to clot than controls, confirming the result of Kellaway and LeMessurier (1935). The venom was also shown to contain hyaluronidase and protease activities (Temple-Smith, 1973).

The work of Temple-Smith (1973) has contributed to our understanding of the seasonal activity of the crural gland and has identified lethal, cutaneous and enzymatic activities of the venom. However, none of the constituents have been structurally characterised and their physiological and pharmacological properties remain unknown. After 30 years, the conclusion of a review on the venomous characteristics of *O. anatinus* by Calaby (1968), that "The whole subject is in need of further study with modern techniques and against the background of modern knowledge of animal venoms.", is still as valid now as it was then.

Pain and oedema are the most consistent and salient feature of *O. anatinus* envenomation. Therefore, the identification and characterisation of components which are likely to produce these effects is of paramount interest and was the major objective of this study. In the next section, I therefore review current knowledge on the mechanisms of pain production by inflammatory mediators and toxins and, in the light of this, consider potential modes of action of *O. anatinus* venom and how these are experimentally addressed in this study.

Pain-producing and pro-inflammatory effects of inflammatory mediators and toxins

Pain is a multicomponent syndrome which encompasses (i) pain directly associated with tissue damage and inflammation, (ii) hyperalgesia, a heightened sensitivity to noxious stimuli; and (iii) allodynia, the induction of pain by normally innocuous stimuli (Woolf *et al.*, 1996). The sensation of pain arises from (noxiously) stimulated activity at pain-sensing afferent endings, or nociceptors, which is conducted along afferent axons that enter and terminate in the dorsal horn of the spinal cord. These terminals form synapses with spinal neurones which give rise to ascending axons that project, *via* the neospinothalamic and paleospinothalamic tracts, to the thalamus which, in turn, projects to the primary somatosensory cortex (Brown, 1989).

A variety of substances produce pain, hyperalgesia and allodynia. Such substances, or algogens, may have an endogenous or exogenous origin. Endogenous algogens consist largely of inflammatory mediators that act primarily on nociceptors. Exogenously derived algogens are predominantly toxins which may act directly on nociceptors or *via* the release of inflammatory mediators. Consequently, the following discussion deals specifically with the peripheral actions of algogens while central mechanisms (at and ascending beyond the level of the spinal cord) which contribute to these actions will not be considered.

Mechanisms of inflammatory pain: endogenous algogens

Nociceptors are high-threshold sensory receptors which have slowly-conducting unmyelinated (C-) or myelinated (Aδ-[4]) parent nerve fibres (Chahl, 1979). They generally respond to more than one of the following stimulus modalities: mechanical,

[4] It has been demonstrated that a phenotypic switch can occur in myelinated Aβ neurones, such that they also mediate inflammatory pain signalling (Neumann *et al.*, 1996).

thermal and chemical. Those responding to all three are termed polymodal nociceptors and make up a high proportion of C-fibre nociceptors.

Algogenic inflammatory mediators in many cases have been shown to either generate action potentials in or to sensitise polymodal nociceptors (Chahl, 1979). Nociceptor activation may arise from changes in nociceptor membrane ion channel activity either, directly, by the action of algogens on receptor-gated ion channels or, indirectly, *via* intracellular second messengers (Rang *et al.*, 1991). An algogen can sensitise[5] nociceptors to the actions of other algogens in several ways: by summation of subthreshold depolarisations; increasing membrane resistance by, for example, blocking K^+ channels; by inducing phosphorylation of intracellular enzymes or ion channels; and by directly modifying the ion channels which underlie the action potential such that their threshold for activation is lowered or inactivation kinetics altered (Bevan, 1996).

Following infection or tissue injury, inflammatory responses are generally initiated and mediated by cytokines[6] which are released from phagocytic cells of the immune system (Rang *et al.*, 1991). This ultimately leads to the liberation of algogenic *inflammatory* mediators by the action of enzymes (i) or secretion from specialised inflammatory cells (ii). In addition, algogenic *neurogenic* factors may be released from sensory neurones (iii) or from sympathetic neurones (iv). Adenosine triphosphate (ATP) (v) may also be released from lysed cells following tissue damage. Additionally, the proton (vi) concentration may be elevated by the increase in anaerobic glycolysis which accompanies inflammation (Kress and Reeh, 1996) and the active transport of lactic acid into the interstitial space by leucocytes (McCarty *et al.*, 1966).

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[5] Sensitisation, in this context, refers to an enhancement of the response or lowering the threshold for firing of nociceptors

[6] Cytokines are a group of low molecular weight proteins which may either stimulate or inhibit the proliferation of immune cells or their secretion of antibodies or additional cytokines (Kuby, 1992)

Both ATP and protons are algogenic. The release of each of these algogenic mediators and their interaction with sensory nerve terminals is summarised in Fig. 1.1 and described in the following sections. It has been the subject of several recent reviews, including those of Rang *et al.* (1991), Kress and Reeh (1996) and Senba and Kashiba (1996).

(i) Enzymatic release of algogenic inflammatory mediators

The two major enzymatic products which play key roles in the pain and hyperalgesia that accompanies tissue damage and inflammation are the eicosanoids[7] (leukotrienes, diHETE (dihydroxyeicosatetraenoic acid) and prostaglandins) and bradykinin.

Eicosanoids appear to play a major role in nociceptor sensitisation and either act directly on nociceptive neurones or stimulate the release of other, directly acting, eicosanoids. Leukotrienes D_4 (LTD_4) and B_4 (LTB_4), released during inflammation *via* the lipoxygenase metabolic pathway, stimulate the synthesis and release of prostaglandins (PGs) and (18R, 15S)-diHETE from polymorphonuclear leukocytes (Rang *et al.*, 1991; Levine *et al.*, 1986; see Fig. 1.1). The prostaglandins PGE_2 and PGI_2 sensitise nociceptive neurones to heat (Mizumura *et al.*, 1993), mechanical stimulation (Mizumura *et al.*, 1987) and to other algogens, for example, bradykinin (Mizumura *et al.*, 1991). In sensory neurones *in vitro* which express nociceptor properties, PGE_2 both increases the magnitude and decreases the activation threshold of the tetrodotoxin-resistant Na^+ current (TTX-R I_{Na}) (Gold *et al.*,1996b). TTX-R I_{Na} is likely to determine the action potential threshold in nociceptors as these neurones have a resting potential at which TTX-sensitive channels (but not TTX-R I_{Na}) are inactivated (Caffrey *et al.*, 1992; Gold *et al.*, 1996b) and this mechanism may thus underlie nociceptor sensitisation by PGE_2 *in vivo* (Gold *et al.*, 1996b). diHETE also produces

[7] Eicosanoids are unsaturated fatty acid derivatives of arachidonic acid

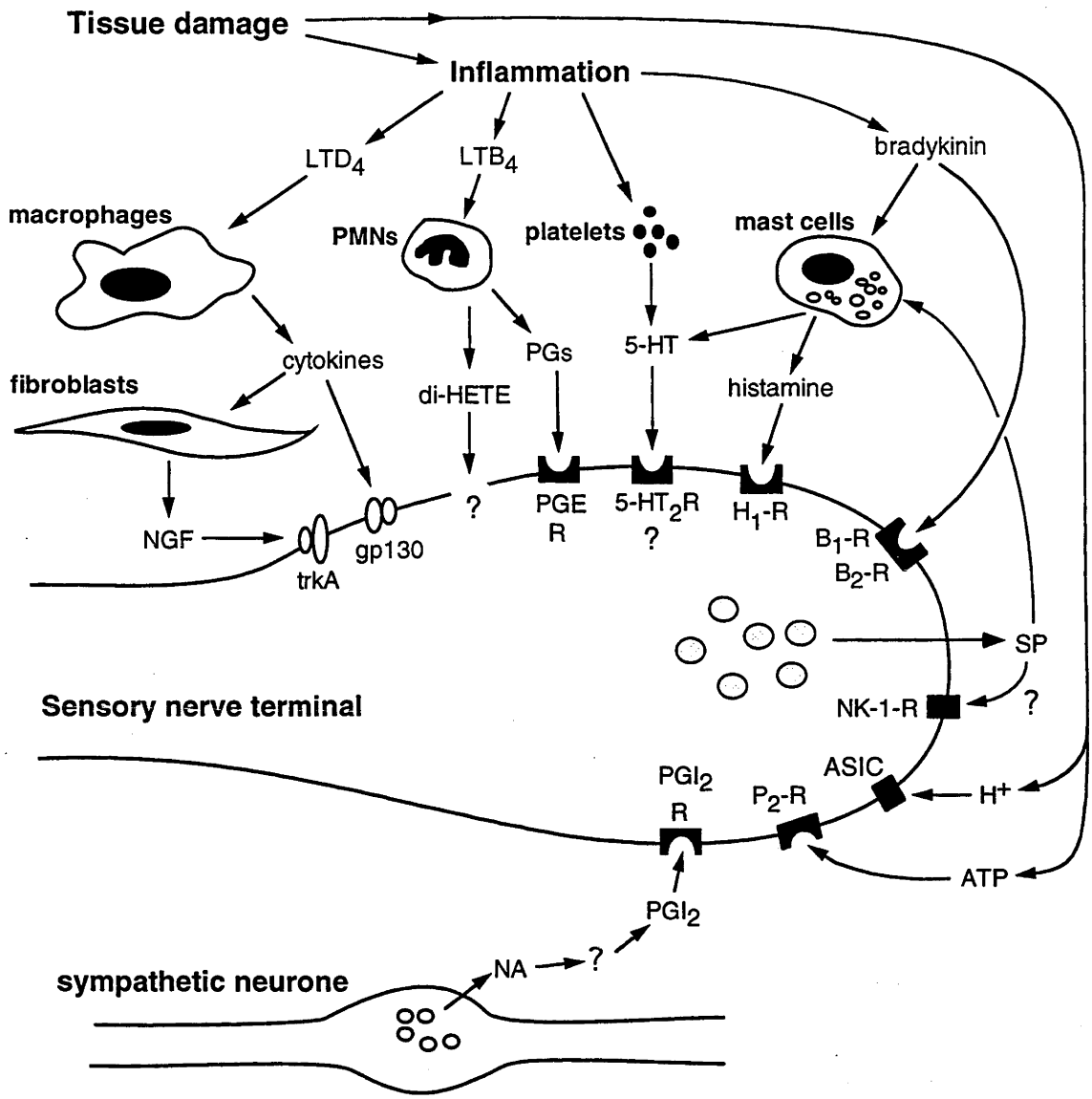


FIG 1.1. Schematic summary of the release of inflammatory mediators and their interactions with sensory nerve terminals. Abbrieviations: LTD_4, leukotriene D_4; LTB_4, leukotriene B_4; PGs, prostaglandins; PMNs, polymorphonuclear cells; NGF, nerve growth factor; 5-HT, 5-hydroxytryptamine; SP, substance P; CGRP, calcitonin gene-related peptide; ASIC, acid-sensitive ion channel; di-HETE, di-hydroxyeicosatetraenoic acid. NA, noradrenaline. Modified from Rang *et al.* (1991) and Senba and Kashiba (1996).

thermal and mechanical sensitisation of nociceptive neurones (White *et al.*, 1990). From these findings it appears that the role of eicosanoids is one of sensitisation rather than nociceptor activation *per se*.

Algogenic kinins, such as bradykinin and kallidin, are also released during inflammation through the action of kallikrein, a proteolytic enzyme. Kallikrein releases kinins from high-molecular weight kininogens which are present in circulating plasma and interstitial fluid (Kress and Reeh, 1996). Bradykinin is a potent producer of pain and hyperalgesia when injected intradermally (Manning *et al.*, 1991) and both polymodal C-fibre and Aδ nociceptors respond to bradykinin (Lang *et al.*, 1990), probably through B2 receptors (Steranka *et al.*, 1988). However, both B1 and B2 receptor antagonists reduce the hyperalgesia induced by Complete Freund's Adjuvant (CFA) and cytokines, suggesting the involvement of both receptors (Davis and Perkins, 1994; Perkins *et al.*, 1995). In cultured sensory neurones, bradykinin activates phospholipase C, thereby elevating IP_3 and DAG levels. DAG in turn activates protein kinase C (PKC), resulting in both Na^+ channel activation and Ca^{2+} influx (Burgess *et al.*, 1989). Aside from its direct effect on nociceptive neurones, bradykinin may act indirectly by degranulating mast cells (Cross *et al.*, 1997) thereby releasing histamine and 5-hydroxytryptamine (5-HT), both of which may activate nociceptive terminals (see below).

(ii) Release of algogens from inflammatory cells

Cytokines trigger the migration of various inflammatory cells through blood vessels into tissue spaces (Kuby, 1992). These cells include platelets, mast cells, polymorphonuclear lymphocytes and macrophages, which all have the potential to release algogenic mediators (Senba and Kashiba, 1996). Fibroblasts also appear to play

an important role as they synthesise and secrete nerve growth factor (NGF), a potent algogen, in response to cytokines (Yoshida and Gage, 1992).

Platelets, which accumulate in tissue spaces during inflammation, release 5-HT upon activation by collagen and platelet-activating factor (PAF, a mast cell-derived secondary mediator) (Kress and Reeh, 1996). 5-HT has been demonstrated both to activate and sensitise nociceptors (Rang *et al.*, 1991). 5-HT_3 receptor antagonists block hyperalgesia associated with carrageenan-induced inflammation or the injection of a combination of inflammatory mediators (Escalier *et al.*, 1989; Kress and Reeh, 1996). This suggests that 5-HT_3, which represents the only ion channel among 5-HT receptors (Kress and Reeh, 1996), is a common target for inflammatory mediators. However, 5-HT_1 and 5-HT_2 receptors have also been implicated in the sensitising and excitatory effects of 5-HT (Rueff and Dray, 1993). Also, 5-HT has been shown to increase the magnitude of TTX-resistant Na^+ currents in sensory neurones *in vitro* (Gold *et al.*, 1996b; Cardenas *et al.*, 1997), by a mechanism which involves 5-HT_4-like receptors (Cardenas *et al.*, 1997). While it is clear from these findings that each of the four 5-HT receptor subtypes plays a role in the nociceptive effects of 5-HT and other mediators, the precise contribution of each subtype remains unclear.

Histamine, in addition to 5-HT in many species, may be released from mast cells following stimulation by the antigen-bound reaginic antibody, immunoglobulin E (IgE) or peptides such as substance P (see following section) (Fig. 1.1). Histamine predominantly produces itch when injected subcutaneously (Keele and Armstrong, 1964) but not pain unless associated with tissue damage (Magerl *et al.*, 1990; Kress and Reeh, 1996). This is reflected in its weak activation of only about 15 % of cutaneous nociceptors (Kress and Reeh, 1996). Sensory neurones express H_1 receptors whose activation by histamine leads to the release of Ca^{2+} from intracellular stores *via* IP_3 (Tani *et al.*, 1990) but it is not known whether this occurs at the sensory terminal *in*

vivo. Histamine may act principally in synergy with other mediators to potentiate their algogenic effects. For example, it has been demonstrated that histamine in combination with bradykinin, 5-HT and PGE_2, has a more than additive influence on the algogenic effects of acidic pH (Steen *et al.*, 1995; Steen *et al.*, 1996; Vyklický *et al.*, 1998).

Cytokines, including interleukins (IL), tumour necrosis factor (TNF) and interferons, are released by phagocytic macrophages and other antigen-presenting cells, which accumulate at the site of tissue injury (Rang *et al.*, 1991). Cytokines of the IL-6 family may have a direct action on nociceptors as gp130, the receptor through which IL-6 members act, is expressed by primary sensory neurones (Banner and Patterson, 1993). However, the possibility that IL-6 may activate or sensitise these neurones has not been investigated. Interestingly, IL-6 itself is also expressed in sensory neurones following nerve injury (Murphy *et al.*, 1995), suggesting a possible autocrine function.

Another cytokine, IL-1β, which is released from macrophages, promotes the secretion of nerve growth factor (NGF) from fibroblasts (Yoshida and Gage, 1992, see Fig 1.1). Aside from its role during development, recent evidence suggests that NGF plays a key role in hyperalgesia. For example, the inflammatory hyperalgesia produced by injecting complete Freund's adjuvant (CFA) into rats is blocked by the prior systemic administration of anti-NGF serum (Woolf *et al.*, 1994). In addition, NGF itself produces lasting hyperalgesia when injected into human subjects (Petty *et al.*, 1994; Dyck *et al.*, 1997) and experimental animals (Lewin *et al.*, 1993b). This activity appears to involve central sensitisation (following retrograde transport of NGF) as well as release of mediators from inflammatory cells and sympathetic neurones (Lewin *et al.*, 1994; Woolf *et al.*, 1996). NGF has also been shown to sensitise visceral Aδ- and C-fibre afferents (Dmitrieva and McMahon, 1996), suggesting a direct action on nociceptors. In addition, the up-regulation of preprotachykinin A (the substance P

precursor) expression in sensory ganglion neurones during CFA-induced inflammation was prevented by treatment with anti-NGF antibodies (Leslie *et al.*, 1995). This suggests that NGF mediates changes in sensory neuronal gene expression during inflammation and it is conceivable that this effect contributes to the longevity of NGF-induced hyperalgesia. NGF has been identified in *O. anatinus* venom in the present study and its hyperalgesic properties are discussed at length in Chapter 6.

(iii) Neurogenic mediators released from sensory neurones: the axon reflex

Nociceptor activation produces a cutaneous flare response, the "axon reflex", which is dependent on intact afferent innervation (Meyer *et al.*, 1996). This is likely to result from the vasodilatory and plasma extravasating effects of substance P and calcitonin gene-related peptide (CGRP) both of which are released from peripheral sensory terminals (Lynn, 1996). As mentioned above, substance P also degranulates mast cells and this may further contribute to the flare response.

In addition to effects on the vasculature and mast cells, it is possible that activated nociceptive terminals release chemicals which activate neighbouring nociceptors. This idea of "spreading sensitisation" was originally suggested by T. Lewis in 1942 but was considered unlikely following the finding that spreading sensitisation to heat does not occur, despite the presence of a reflexive flare response (Meyer *et al.*, 1996). However, it is possible that neurogenic spreading sensitisation to mechanical and chemical stimuli may still occur and could, like the "efferent" effect of sensory neurones on the vasculature, also involve neuropeptide release. Interestingly, several recent studies have demonstrated that cultured sensory neurones express "autoreceptors" for substance P (*i.e.* neurokinin or NK-1 receptors) and the application of substance P or the NK-1 agonist, Sar-SP, to these neurones, produces an inward current and elevates intracellular calcium (Hu *et al.*, 1997; Brechenmacher *et al.*, 1998; Li and Zhao, 1998). Unfortunately, these authors only

considered the possible presynaptic role of these receptors in the dorsal horn of the spinal cord. However, the distribution of expressed substance P autoreceptors may also extend to peripheral terminals of nociceptive neurones and, if so, would provide a possible mechanism for spreading sensitisation.

(iv)Neurogenic factors released from sympathetic neurones

The pain associated with some chronic pain states, such as causalgia and reflex sympathetic dystrophy, is alleviated following sympathectomy suggesting the involvement of sympathetic post-ganglionic neurones (McMahon, 1991). Stimulation of sympathetic efferents has been shown to increase firing in C-fibres (Rang *et al.*, 1991) and noradrenaline increases heat hyperalgesia produced by capsaicin (Drummond, 1995). However, it is unlikely that the effect is simply due to a direct action of noradrenaline released from sympathetic fibres as high noradrenaline concentrations do not activate C-fibers (Lang *et al.*, 1990; Rang *et al.*, 1991). However, prostacyclin (PGI_2) induces hyperalgesia with a similar time-course to noradrenaline, suggesting that PGI_2 may mediate sympathetic neurogenic hyperalgesia (Taiwo and Levine, 1988; Fig. 1.1). Clearly, more work needs to be done to clarify the involvement of sympathetic post-ganglionic neurones in pain and hyperalgesia.

(v) Adenosine triphosphate (ATP)

ATP is present in all cells at millimolar concentrations and large amounts are therefore likely to be released following physical cell damage (Rang et al., 1991). ATP induces pain in humans when applied to a blister base (Keele and Armstrong, 1964). This may be due to a direct effect on nociceptors, as ATP activates a cation channel in sensory neurones *in vitro* (Krishtal *et al.*, 1988), an effect which is blocked by P2-receptor antagonists (Rang *et al.*, 1991).

(vi) Protons

Inflammatory exudates are acidic (McCarty *et al.*, 1966) and acidic solutions (buffered at pH 6.2) produce pain when injected subcutaneously or applied to a blister base (Keele and Armstrong, 1964). Acidic pH directly activates polymodal nociceptors and potentiates the effects of other inflammatory mediators, including bradykinin, 5-HT, histamine, and PGE_2 (Steen *et al.*, 1995). Recent evidence suggests this facilitatory action is mediated through capsaicin receptors (Vyklický *et al.*, 1998; see next section). In sensory neurones, acidic pH evokes both transient and sustained inward cation-selective currents (Bevan, 1996) possibly *via* an acid-sensitive ion channel (ASIC) recently found to be expressed in these neurones (Waldmann *et al.*, 1997). The sustained current is markedly potentiated in the presence of an inflammatory mediator "cocktail" (containing bradykinin, 5-HT, PGE_2 and histamine), further supporting the notion that a synergy exists between these mediators and acidic pH (Kress *et al.*, 1997).

General comments

It is clear that a given inflammatory mediator is unlikely to act alone on nociceptive neurones following tissue injury. In many of the aforementioned examples, combinations of mediators act synergistically to produce a suprathreshold response. An interaction between inflammatory mediators and physicochemical stimuli, such as low pH, is also very likely. A sustained potentiation of the painful effects of tissue acidosis is seen when inflammatory mediators are combined with low pH (Steen *et al.*, 1996). This could explain a major anomaly between the marked desensitisation observed following prolonged administration of individual algogenic mediators and the longevity of inflammatory pain (Kumazawa *et al.*, 1987; Lang *et al.*, 1990; Vyklický *et al.*, 1998), *i.e.* prolonged algetic responses may result from a combination of

inflammatory mediators and tissue acidosis, rather than from the effects of individual components.

Toxins which produce pain: exogenous algogens

Snake and arthropod venoms

In general, there are four[8] ways by which several of the aforementioned mechanisms and pathways underlying inflammatory pain can be triggered following envenomation.

(i) Venom components may liberate algogens from endogenous precursors. For example phospholipases A_2, which are present in almost all snake (Iwanaga and Suzuki, 1979), as well as bee (O'Conner and Peck, 1979), wasp (Edery *et al.*, 1979) and scorpion (Ramanaiah *et al.*, 1990) venoms, cleave the C_2 acyl chain of phosphatidylcholine, thereby generating arachidonic acid and lysophosphatidylcholine. Arachidonic acid is converted to prostaglandins through the action of cyclooxygenases. As described above, prostaglandins sensitise nociceptors whereas lysophosphatidyl-choline is a mast cell degranulator (Moreno *et al.*, 1992) and may be converted to platelet-activating factor (PAF) (Teixeira *et al.*, 1994). Certain snake venoms also contain kallekrein-like enzymes which liberate bradykinin from high molecular weight kininogens (Iwanaga and Suzuki, 1979).

(ii) Venoms may elicit an antigenic response, leading to the release of inflammatory algogens. For example, IL-6, which may have a direct effect on nociceptive neurones, is released following *Bothrops* ssp. and Crotalid snake envenomation and scorpion sting in humans (Sofer *et al.*, 1996; Barraviera *et al.*, 1995). An investigation into the behavioural hyperalgesia induced by *Bothrops jararaca* venom in rats has also demonstrated the involvement of eicosanoids and PAF

[8] pain which is secondary to necrotic and myotoxic effects of venoms is not considered here.

(Teixeira *et al.*, 1994).

Several insect venoms contain peptides which release histamine and 5-HT from mast cells and in this way may contribute to their painful effects. Examples include melittin from *Apis mellifera* venom (Jasani *et al.*, 1979) and mastoparans from Vespid wasp venoms (Ho and Hwang, 1991).

(iii) Venoms may contain factors which potentiate the activity of exogenous or endogenous algogens. For example, peptides which potentiate the activity of bradykinin are present in snake (*e.g. Bothrops jararacussu*, Ferriera *et al.*, 1992), spider (*e.g. Scaptocosa raptoria*, Ferreira *et al.*, 1996) and scorpion (*e.g. Buthus occitanus*, Meki *et al.*, 1995) venoms. These bradykinin-potentiating peptides act by inhibiting angiotensin-converting enzyme (ACE) which is the major enzyme responsible for the breakdown of bradykinin in the vasculature and other tissues (Campbell, 1995).

(iv) Venoms may themselves contain algogens which act directly on nociceptive neurones. For example, Vespid wasp venoms contain kinins which are structurally and pharmacologically similar to bradykinin (Edery *et al.*, 1978) and may therefore contribute to the painful effects of envenomation. In addition, NGF is present in the venom of snakes from several genera (Kostiza and Meier, 1996) and may act similarly (see above).

It is possible that many venoms which produce pain do so by directly activating or modifying the gating and kinetics of ion channels on nociceptive terminals. For example, versutoxin, from the venom of the Australian funnel-web spider, *Hadronyche versuta*, removes TTX-sensitive Na^+ channel inactivation in DRG neurones (Nicholson *et al.*, 1994) and similar effects are produced by scorpion (*Centruroides sculpturatus* and *Leiurus quinqestriatus*) neurotoxins (Wang and Strichartz, 1983). It is conceivable that such effects on Na^+ channel inactivation contribute to the pain which is a major

presenting symptom following envenomation by these species (Sutherland, 1983; Rimsza *et al.*, 1980).

These examples demonstrate that activation of a number of potentially algogenic pathways by animal venoms does occur and has been found largely to involve the release or potentiation of endogenous inflammatory mediators. However, with the exception of the study by Teixeira *et al.* (1994) described above, no systematic behavioural or electrophysiological studies on the mechanisms of venom or venom-derived-toxin-induced pain and hyperalgesia have been carried out. This is despite the fact that numerous venoms produce pain (Chahl and Kirk, 1979) and this is often a major presenting symptom following envenomation.

In contrast to the situation with animal toxins, the nociceptive properties of the plant-derived neurotoxin, capsaicin, have been studied extensively.

Capsaicin

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the pungent component of capsicum peppers which gives rise to a sensation of warmth and burning. It produces burning pain and mechanical hyperalgesia when injected intradermally (Simone *et al.*, 1989) and selectively activates C-fibre polymodal nociceptive, thermoceptive and Aδ-fibres (Bevan and Szolcsanyi, 1990). Prolonged application is selectively excitotoxic for C-fibres *in vivo* (Jansco *et al.*, 1977) and *in vitro* (Wood *et al.*, 1988). Capsaicin depolarises sensory neurones by activating a non-selective cation current (Baccaglini and Hogan, 1983; Bevan and Forbes, 1988) and also, probably secondarily, increases cGMP, DAG, IP_3 and arachidonic acid in these neurones (Wood *et al.*, 1989; Burgess *et al.*, 1989).

The selectivity of capsaicin for thermo- and nociceptive neurones suggests that capsaicin receptors may form part of an important activation pathway in pain

transduction and has prompted several theories on their normal function. Similarities between the currents evoked by low pH and capsaicin initially led to speculation that capsaicin receptors normally transduce proton-mediated activity (Bevan and Geppetti, 1994; Liu and Simon, 1994). However, the finding that protons did not activate capsaicin-activated channels in outside-out patches of sensory neurones makes this suggestion untenable (Oh *et al.*, 1996). Several recent advances have suggested more plausible hypotheses. The capsaicin receptor, VR1 (vanilloid receptor 1) has recently been identified, cloned and expressed in oocytes (Caterina *et al.*, 1997). Capsaicin activates an inward current only in VR1-expressing oocytes and this is potentiated by low pH (6.3). Transfected cells also exhibit noxious heat-activated inward currents (Caterina *et al.*, 1997). Thus, the normal role of VR1, which is predominantly expressed by small to medium diameter sensory neurones (putative nociceptors), may be to transduce noxious heat signals and mediate nociceptor potentiation by the low pH which accompanies inflammation. This is further supported by the recent findings of Vyklický *et al.* (1998) which suggest that the facilitatory action of low pH on the activity of inflammatory mediators occurs through capsaicin receptors.

Possible bases for the algogenic effects of O. anatinus venom

O. anatinus venom may produce lasting pain through any of the aforementioned mechanisms. Like other venoms, *Oa*V may contain or cause the release of inflammatory mediators. However, the longevity and severity of the symptoms associated with envenomation are not consistent with the acute activity of some mediators, such as histamine, 5-HT and bradykinin, whose effects are moderate and short-lived by comparison (Keele and Armstrong, 1964). Thus while these mediators may contribute to acute effects, others such as NGF, which produces lasting hyperalgesia (Lewin *et al.*, 1993b) and alters gene expression (Leslie *et al.*, 1995), are

more likely to contribute to the long-term effects. In fact, a single subcutaneous dose of NGF in human subjects produces localised hyperalgesia persisting for up to 7 weeks (Petty *et al.*, 1994; Dyck *et al.*, 1997), a time-course similar to that reported following *O. anatinus* envenomation. It is also possible that *Oa*V has a selective, direct effect on nociceptive neurones, in an analogous manner to capsaicin.

Objectives of this study

The studies described in this thesis were ultimately aimed at identifying and characterising components from *O. anatinus* venom which contribute to the painful and pro-inflammatory effects of envenomation. The formulation of experiments to meet this objective presented a number of difficulties. Firstly, pain is a subjective experience and as such is difficult to measure experimentally. Behavioural testing, *e.g.* measuring the limb withdrawal latencies of experimental animals in response to noxious mechanical or thermal stimuli, may be used as an index of pain. However, this technique relies on the assumption that animals behave in such a way in response to pain as humans subjectively experience it and is therefore, by definition, anthropomorphic. Anthropomorphism does not present the same problem in other types of animal experimentation, *e.g.* in physiological experiments, because animal and human parameters in these cases can be objectively compared and the validity of an animal model may thus be reasonably assessed. However, a reflexive response to a noxious stimulus provides no information on the subjective sensation of pain likely to be experienced by the animal. Although it could equally be argued that data obtained from humans presents a similar problem (pain is still subjective), human subjects have the advantage of being able to communicate the sensations experienced in a complex manner. This allows a more valid comparison of reported sensations with our own and those of other subjects. Pain may be measured in this way, following administration of

an algogen to human subjects, using subjective rating scales (Keele and Armstrong, 1964). However, this presents a number of additional problems. For example, apart from obvious ethical implications for severely painful stimuli, the amount of detailed mechanistic information which may be obtained is very limited.

An alternative to the behavioural approach is to assay for the venom-induced release of inflammatory mediators which are implicated in the induction of pain. Concomitantly, the possibility of a direct effect of venom components may be addressed by measuring the activity of pain-sensing neurones. Both types of experiment were employed in this study, with a particular emphasis on the latter.

Because very little was known about the characteristics of venom constituents at the commencement of this work, an initial part of this study involved the purification and characterisation (at the amino acid sequence level) of the major venom proteins and peptides (Chapter 3). One peptide, a C-type natriuretic peptide, was extensively characterised and demonstrated to release histamine from purified rat peritoneal mast cells and to produce some pro-inflammatory effects which are characteristic of *O. anatinus* venom (Chapter 4). However, an exhaustive analysis of the release of inflammatory mediators by venom components and their contribution to the painful effects of envenomation was not undertaken. Rather, the possibility of a direct effect on sensory neurones was investigated using sensory ganglion neuronal cell bodies *in vitro*, which express properties and chemical sensitivities similar to polymodal nociceptors *in vivo* (Chapter 5). *O. anatinus* venom did indeed directly activate inward currents in these neurones and this activity was associated with an NGF-like protein which was characterised further (Chapter 6).

CHAPTER 2

GENERAL METHODS

The general methods used in the following experimental chapters are described here. Specific variations and methods confined to a single chapter are detailed in the Methods section of that chapter.

Collection and handling of O. anatinus venom

The majority of material used in this study was obtained from excised crural glands, which were a gift from Dr Russell Jones (University of Newcastle, School of Biological Sciences). Animals (3), trapped in August from Barrington Tops (NSW), were sacrificed by pentobarbitone overdose. A ligature was tied at the distal end of the main duct to prevent the loss of material and each crural gland-duct complex was dissected free and placed in frozen CO_2. Crural glands were subsequently stored at -70 °C and thawed at 4 °C when required.

Material was obtained from the glands using a similar method to that described by Temple-Smith (1973). An incision was made in the duct just above the ligature and material was collected by holding the crural gland and allowing the duct to hang vertically into a 1.5 ml polypropylene tube placed on wet ice. Material spontaneously leaked from the incision but additional material could be obtained by gently applying pressure to the crural gland. The collected venom was stored neat in aliquots at -70 °C. Lyophilisation has been reported to produce substantial losses in material and activity (Temple-Smith, 1973) and was therefore avoided.

Additional material was collected by Dr Melody Serena (Australian Platypus Conservancy, Whittlesea, VIC) from animals which were captured and released during

routine field surveys. This was accomplished by holding the spur at right angles to the hind-limb and aspirating droplets of venom, which appeared on the tip of each spur, using a capillary-tipped Gilson micropipette. The animals were released with no apparent ill effects and the material was placed on ice in the field and stored long-term at -20 °C. This had been determined to be the most effective, rapid and innocuous method of obtaining material from live animals during previous field trips with Dr David Goldney (Charles Sturt University, Bathurst, NSW) (de Plater, Hons Thesis, ANU 1993). Attempts to collect material using latex stretched over a collection reservoir (analogous to the method used for the collection of snake venoms), the injection of oxytocin (commonly used on field trips to facilitate lactation in females), or mild trans-cutaneous electrical stimulation, proved to be unsuccessful.

High-performance liquid chromatography (HPLC)

Prior to each HPLC step, a thawed aliquot of *O. anatinus* venom was suspended in the eluting buffer for isocratic separations and eluent A (see next section) for gradient separations. This invariably resulted in the formation of a white precipitate which was removed by centrifugation. The precipitation appeared to be more pronounced in Tris- (pH 8.5) or HEPES- (pH 7.5) buffered solutions and less pronounced in ammonium acetate- (pH 5.0) and, particularly, MES- (pH 6.1, with sorbitol-see below) buffered solutions.

Reversed phase HPLC (RP-HPLC)

For the reversed phase separation of peptides of less than 10 kDa, venom was suspended in 0.1 M ammonium acetate, pH 5.0, clarified by centrifugation and subjected to ultrafiltration using a 10 kDa nominal molecular weight filter (Centricon 10, Amicon or Ultrafree 10 k NMWL, PTGC, Waters). The filtrate was recovered and fractionated by reversed phase HPLC (RP-HPLC) using a C2/C18 column of dimensions 2.1 mm x 10

cm (μRPC SC2.1/10, Pharmacia) or 3.2 mm x 3 cm (μRPC PC 3.2/3, Pharmacia) connected to a SMART (Pharmacia) HPLC system. Eluent A consisted of 0.1 % trifluroacetic acid (TFA) in H_2O and eluent B consisted of 0.1 % TFA in CH_3CN. Peptides were eluted using a linear gradient of 5-60 % B over 20 min at a flow rate of 250 ml min^{-1}. The eluate was monitored continuously using a μPeak detector (Pharmacia) at wavelengths of 214, 255 and 280 nm unless indicated otherwise.

In some experiments, whole *O. anatinus* venom or fractions from other HPLC steps were subjected to RP-HPLC. The conditions were as described above except that the material was suspended in 0.1% TFA, 5% CH_3CN and not subjected to ultrafiltration. Detection was at 214, 255 and 280 nm unless indicated otherwise.

Ion-exchange HPLC

Whole venom was fractionated by cation-exchange HPLC using a MonoS PC 1.6/5 (1.6 x 50 mm, Pharmacia) column connected to the SMART System and eluted with a 0-1 M NaCl gradient in 50 mM HEPES, pH 7.5. In some experiments 20 mM MES, pH 6.1, was used. Fractions were further purified by RP-HPLC as described above, following titration to approximately pH 3 with 3 M TFA. Material not binding to the cation-exchange column was fractionated by anion-exchange HPLC using a MonoQ PC 1.6/5 (1.5 x 50 mm, Pharmacia) column with a linear gradient of 0 - 0.5 M NaCl in 20 mM bis-Tris, pH 6.5.

Gel permeation HPLC (HP-GPC)

HP-GPC on the SMART System was carried out using a Superose-12 (Pharmacia) agarose-based column of dimensions 3.2 mm x 300 mm. The column was equilibrated with several volumes of 50 mM ammonium acetate (pH 5.0) prior to each run. The sample was injected in a minimal volume (< 50 μl) and eluted using a flow rate of 40 μl min^{-1}. In some experiments, MES buffer (mM, 135 NaCl, 3 KCl, 0.6 $MgCl_2$, 2.5 $CaCl_2$,

1.2 $NaHCO_2$, 10 glucose, 10 MES, titrated to pH 6.1 with HCl, osmolarity adjusted to 320 mOsm using sorbitol) was used. The eluate was monitored continuously at 280 nm.

Chromatofocusing

Chromatofocusing was carried out using a MonoP HR 5/20 column (Pharmacia). This column, designed for use with FPLC (Pharmacia) systems, was adapted for use with the SMART System using a long column adaptor and incorporating several reservoir fill commands in the gradient program (the volumes required exceed the capacity of the SMART buffer reservoir cylinders). The column was equilibrated in 0.025 M triethylamine which was adjusted to pH 11.0 with HCl (buffer A). The eluant consisted of 1 part Pharmolytes 10.5 - 8.0 (Pharmacia, undiluted) to 45 parts MilliQ H_2O (buffer B). Both the equilibrating and eluting buffers contained 55 mM sorbitol to facilitate analyte solubility. Following sample injection, the equilibrating buffer was run through the column at a flow rate of 0.5 ml min^{-1} for 20 min. The eluting buffer (*i.e.* 100% B) was then run through the column for 100 min at a flow rate of 0.5 ml min^{-1}. The pH of the eluate was monitored by measuring the pH of collected fractions at regular intervals. Ampholytes were largely removed from chromatofocusing fractions by ultrafiltration (Ultrafree 10 k NMWL, PTGC, Waters) and HP-GPC.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Vertical slab sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under non-reducing conditions using Novex 10-20 % gradient pre-cast gels in a Novex Xcell Mini-Cell (Novel Experimental Technology, San Diego, CA, USA) and a *N*-Tris(hydroxymethyl)aminomethane-*N*-Tris(hydroxymethyl)methylglycine (Tris-Tricine) (Schågger and von Jagow, 1987) or Tris-Glycine (Laemmli, 1970) buffer system. Samples were denatured by heating in sample buffer (0.16 M Tris-HCl, pH 8.45, 4 % (w/v) SDS, 20% (v/v) glycerol + tracking dyes) at 100 °C for 10 min prior to

application. Gels were run at a constant voltage of 125 V (current: 80 mA/gel start, 40 mA/gel end) for approximately 90 min. Proteins were visualised using 0.1 % Coomassie blue R250 in 10 % propan-2-ol, 10 % acetic acid. Silver staining was carried out according to a protocol based on Blum *et al.*, 1987. Gels were fixed by incubation in 50 % ethanol, 12 % acetic acid for 15 min followed by 30 % ethanol for 15 min. The fixing solution was decanted and the gel incubated for 1 min in 0.8 mM sodium thiosulphate. The gel was washed 3 times for 30 s in MilliQ H_2O and incubated in 14 mM $AgNO_3$, 0.075 % formaldehyde. The gel was again washed in H_2O and developed using 0.57 M $NaCO_3$, 0.16 mM sodium thiosulphate and 0.05 % formaldehyde. Staining was stopped by replacing the developing solution with 30 % ethanol, 12 % acetic acid.

Amino acid analysis

Venom and peptide concentrations were determined by quantitative amino acid analysis. Lyophilised samples were subjected to gas-phase hydrolysis under nitrogen at 110 °C for 20 h, using 6 N HCl with 0.1 % phenol. Analyses were performed using a Hewlett-Packard AminoQuant Series II (Hewlett-Packard) amino acid analyser. Amino acids were subjected to pre-column derivitisation with *ortho*-phthalaldehyde (OPA with 3-mercaptopropionic acid in 0.4 M sodium borate, pH 10.4, Hewlett-Packard) and 9-fluorenylmethyl chloroformate (FMOC) and derivitised amino acids were detected using an HP 1046 (Hewlett-Packard) fluorescence detector. Quantitative analyses were performed in triplicate using a three-level calibration for each amino acid.

Mass spectrometry

Peptide molecular masses were determined using a VG Analytical TOFSPEC (Time-of-Flight, Matrix-Assisted Laser Desorption and Ionisation, TOF-MALDI) instrument (VG Analytical Division, Fisons Instruments, Manchester, U.K.) and a saturated solution of

α-cyano-4-hydroxycinnamic acid (in 40% CH_3CN/0.1% trifluoroacetic acid) as matrix. Masses were determined at threshold laser energy using bovine insulin (Sigma, $[M+H]^+$ = 5734.5) and gramicidin S (Sigma, $[M+H]^+$ = 1142.5) as internal reference standards. Fast Atom Bombardment (FAB) and atmospheric pressure ion-spray (APSI) mass spectrometry was performed by Carl Braybrook (Research School of Chemistry, ANU) using VG Analytical ZAB SEQ and QUATTRO instruments, respectively. Samples were prepared in α-thioglycerol for FAB and in 1% formic acid/H_2O for APSI.

N-terminal amino acid sequencing

Purified peptides and proteins were reduced and *S*-pyridylethylated by incubation in 6 M guanidine-HCl, 0.5 M Tris-HCl, pH 7.9, 1 mM EDTA and 10 mM DL-dithiothreitol for 1 h at 37 °C. 20 mM 4-vinylpyridine (Sigma) was added and the solution incubated at room temperature for 2 h. Pyridylethylated peptides were desalted by RP-HPLC and passively adsorbed onto polyvinylidene difluoride (PVDF) membranes (Problott™, Applied Biosystems Division, Perkin-Elmer Corp.), treated with Biobrene™ (Applied Biosystems Division, Perkin-Elmer Corp.) and subjected to automated Edman degradation using Applied Biosystems 494 Procise HT and cLC pulsed liquid protein sequenators (Applied Biosystems Division, Perkin-Elmer Corp.).

Endoproteolytic cleavage of proteins and peptides for Edman sequencing

In-solution cleavage

RP-HPLC-purified, reduced and *S*-pyridylethylated (4-vinylpyridine, Sigma) peptides and proteins were lyophilised and resuspended in 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.6, containing 2 % (w/w, enzyme:substrate) endoproteinase Arg-C (sequencing grade, Boehringer Manheim Biochemica, with 50 mM EDTA and 50 mM dithiothreitol) or 0.1 M NH_4HCO_3 containing 1 % (w/w) chymotrypsin (sequencing grade, Boehringer

Manheim Biochemica). Arg-C digests were incubated for 3 h at 37 °C and chymotryptic digests at room temperature for 2 h and the resulting cleavage products were purified by RP-HPLC and analysed by MALDI-TOF mass spectrometry as described above. In some experiments, endoproteinase Asp-N (sequencing grade, Boehringer Manheim Biochemica) was used at a concentration of 2 % (w/w) for 1 h at 37 °C in 50 mM sodium phosphate, pH 8.0.

In-gel tryptic cleavage

The following method, which was based on Jeno *et al.* (1995) and Hellman *et al.* (1995) was used for the *in situ* reduction, alkylation, and enzymatic digestion of proteins in-gel.

Gel pieces, containing Coomassie-stained bands, were excised and destained with two 250 µl washes of 40% n-propanol for 5 min followed by two extractions with 250 µl of 0.2 M NH_4HCO_3 / 50 % acetonitrile for 15 min at 30 °C. Residual bicarbonate and acetonitrile were evaporated in a vacuum centrifuge. Reduction was carried out prior to enzymatic digestion in a total volume of 50 µl containing 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1 mM EDTA, 10 mM DTT for 45 min at 60 °C. After cooling to room temperature, alkylation was achieved by adding 4-vinylpyridine to a final concentration of 50 mM followed by incubation for 15 min at room temperature. The gel piece was washed several times with 0.2 M NH_4HCO_3 / 50 % acetonitrile and dried in a vacuum centrifuge.

Digestion was initiated by adding 5 µl of 0.2 M NH_4HCO_3 followed by 0.5 µg of porcine trypsin ("modified trypsin sequencing grade" from Promega Corp., Madison, WI), dissolved in 1 mM HCl at a concentration of 0.1 mg ml^{-1}. The gel piece was then fully reswollen by repeated additions of 5-10 µl aliquots of 0.2 M NH_4HCO_3 and the mixture incubated overnight at 30 °C. To terminate the digestion, a 1/10 volume of 10 % TFA was added and the supernatant collected. The resulting peptides were extracted

from the gel pieces by two treatments for at least 40 min each with 100 µl of 0.1% TFA, 60 % acetonitrile at 30 °C. The extracts were combined and the organic phase volume was reduced using a vacuum centrifuge, and the peptides were purified by RP-HPLC as described above.

CHAPTER 3

GENERAL COMPOSITION AND CHARACTERISATION OF O. ANATINUS VENOM CONSTITUENTS

Introduction

It has been demonstrated previously that *O. anatinus* venom (*Oa*V) contains at least seven proteins, which were classified as pre- (M_r[9] < 70 kDa) or post-albumins (M_r 70-80 kDa), or larger (M_r > 90 kDa) proteins (Temple-Smith, 1973). However, no specific information, such as individual molecular weight estimates or amino acid sequence data were obtained for any of these proteins. In addition, no attempt was made to identify and characterise smaller peptides or other non-protein components in *Oa*V. Compositional differences between *Oa*V obtained from excised crural glands and that obtained from live animals has also not been investigated. The work presented in this chapter was aimed at providing M_r estimates and amino acid sequence data for the major *Oa*V proteins and polypeptides as well as resolving any variation between *Oa*V collected by different methods and at different times of year.

Although the results reported by Temple-Smith (1973) suggested that seasonal variation occurs in the relative expression of the three classes of protein identified in *Oa*V, variation of expression of individual components was not clearly established. In addition, seasonal variation was not distinguished from variation due to other factors. Snake venom composition, for example, may vary at the intraspecies level, between individual specimens, due to geographical variation and, in individual specimens, due to seasonal variation, diet, habitat, and age (Chippaux *et al.*, 1991). Fortunately, the

[9] M_r, relative molecular mass

*Oa*V used in this study (supplied by Dr Melody Serena), was obtained at the same locale, from animals which were at least 3 years of age and from several individuals at each collection time. This allowed seasonal variation to be distinguished from variation due to most of the aforementioned factors.

In snake venom research and antivenom production, pooled material is invariably used which yields an average venom quality and may be considered a statistically representative "standard" (Kalapothakis and Chavez-Olortegui, 1997). The definition of a "standard" *Oa*V composition and the characterisation of individual components may be useful for ascertaining *Oa*V variation and for future reference. Such a standard has been prepared in this study by pooling *Oa*V from several individual specimens.

The characterisation of individual components, particularly at the amino acid sequence level, may be employed to obtain an indication of function based on homology with previously characterised proteins. Obtaining amino acid sequence "tags" for individual *Oa*V components will facilitate the future cloning and expression of *Oa*V proteins from an *Oa*V cDNA library, which may become necessary due to the difficulty of obtaining viable quantities of material.

This study describes the general compositional characteristics of pooled *Oa*V samples derived from live animals and excised glands as well as individual and seasonal variation in the expression of *Oa*V proteins. Purification strategies for the major venom polypeptides have been devised and are described along with the partial or full amino acid sequences where these have been obtained.

Methods

HPLC purification and SDS-PAGE analysis of OaV components

Reversed phase, ion exchange, and gel permeation HPLC were carried out using the SMART HPLC System as described in Chapter 2 (General Methods). Material was

obtained from excised crural glands (*Oa*VA, collected in August and pooled) or directly from live animals (*Oa*VB, collected in January, June, September and October and pooled, but also analysed individually) and was analysed by SDS-PAGE using both the Tris-Tricine and Tris-Glycine buffer systems, as described in Chapter 2.

N-terminal Edman sequencing

Purified *Oa*V peptides and proteins were subjected to direct N-terminal Edman sequencing following passive adsorption onto Biobrene-treated PVDF or TFA-activated glass-fibre disc. Some *Oa*V components were subjected to in-gel or in-solution proteolytic digestion, followed by RP-HPLC (using a Sephasil C18 column, Pharmacia) of the resulting peptide fragments which were adsorbed onto PVDF and submitted to Edman sequencing (see Chapter 2).

Database search protocols and algorithms

Amino acid sequences were analysed for similarity to known proteins using both the Baylor College of Medicine (BCM) Search Launcher (URL: http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html) and the Australian National Genome Information Service (ANGIS, http://www.angis.org.au). Both BLAST (Basic Local Alignment Search Tool, Altschul *et al.*, 1990)/BEAUTY (BLAST Enhanced Alignment Utility, Worley *et al.*, 1995) and FastA (Pearson and Lipman, 1988) search algorithms were used. The following databases were searched: dbEST (Expressed Sequence Tags), a database of cDNA sequences which have been reverse-transcribed from mRNA; SWISS-PROT (annotated database maintained by the Dept. Medical Biochemistry at the University of Geneva); and NR (non-redundant), a protein sequence database collated from other databases (GenPept, TREMBL, SWISS-PROT and PIR) and screened to remove redundancies.

Gross similarities between proteins, recognised by a particular reference sequence, were determined using PROSITE, a database of biologically significant sites, patterns and profiles in proteins (ExPASy, Appel *et al.*, 1994). The family and superfamily to which a given partially sequenced *Oa*V protein was likely to belong could be established in this way.

When an *Oa*V protein appeared to be related to a particular class or family, the peptide fragments were aligned individually to members of the family (which were aligned together) using the Clustal W multiple sequence alignment program (Ver. 1.7), part of the BCM Search Launcher (URL: http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

Results

General features of O. anatinus venom

*Oa*V was obtained both from live animals and excised crural glands as a translucent, highly viscous liquid which became turbid upon dilution. The protein concentration of the main *Oa*V stock obtained from excised glands (*Oa*VA) was found to be 39.481 ± 2.656 mg ml^{-1} (mean ± S.D., triplicate analysis) by quantitative amino acid analysis and had a pH of 6.4. Most of the protein components of *Oa*V were identified by SDS-PAGE either using Tris-Glycine (Fig. 3.1A) or Tris-Tricine gels (Fig. 3.1B). Tris-Tricine SDS-PAGE gave a superior separation of venom proteins, particularly in the low molecular weight range and was used routinely for subsequent analyses. Separate analysis of pooled material obtained from excised crural glands (*Oa*VA) and directly from live animals (*Oa*VB) enabled the detection of 11 components, with relative molecular masses ranging from 4-216 kDa (Fig 3.1B). Most components were observed to be present in material from both sources. Differences in the apparent relative concentration of individual components in *Oa*VA and *Oa*VB may

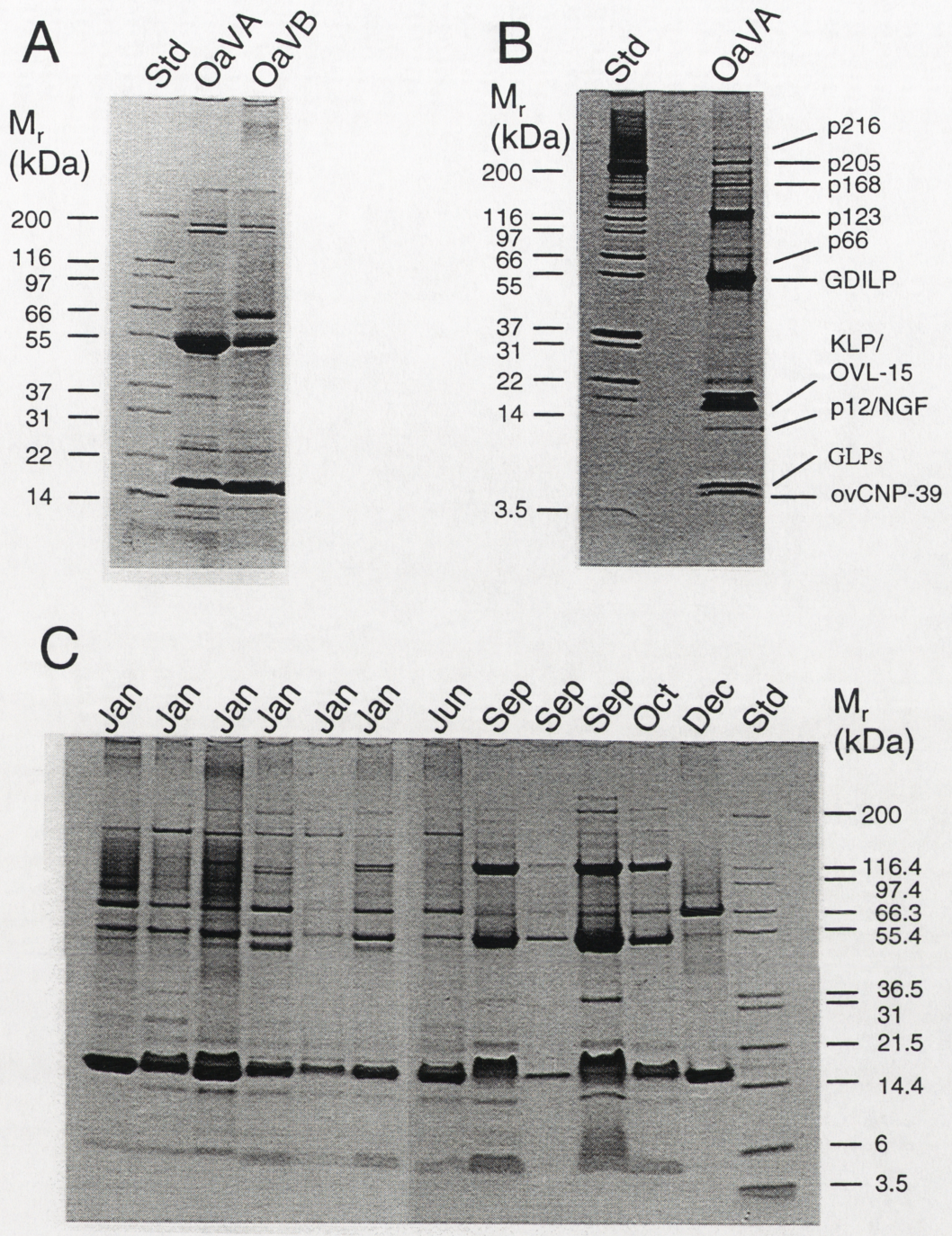


FIG 3.1. A. Tris-Glycine SDS-PAGE analysis of *O. anatinus* venom obtained from excised crural glands (*Oa*VA) and directly from the spurs of live animals (*Oa*VB). For each source *Oa*V from different animals was pooled. **B.** Tris-Tricine SDS-PAGE analysis of *Oa*VA annotated with the names of identified components (see Table 1). **C.** Tris-Tricine SDS-PAGE analysis of OaV samples collected at different times of year, grouped according to the month of collection.

be attributed to a seasonal bias because most of the *Oa*VB material was collected in January and pooled with *Oa*V collected at other times of the year, whereas the entire OaVA pool was obtained in August.

Fractionation by HPLC led to the detection of 19 *Oa*V components; more than by SDS-PAGE, largely due to isoforms co-migrating on SDS-PAGE. These components are described in Table 3.1. Several proteins have been characterised at the amino acid sequence level and their sequences compared with those in the SWISS-PROT, NR and EST databases (BCM and ANGIS). Possible homologies are summarised in Table 3.1. Discrete components have been named according to their molecular weight or according to sequence homologies where these have been established.

In addition to the analysis of pooled samples (*Oa*VA and *Oa*VB) *Oa*V samples collected from different individuals at different times of the year were analysed by SDS-PAGE (Fig. 3.1C). *Oa*V obtained outside the breeding season, where crural glands are in the regression, quiescent (December, January) or elaboration phases (June), respectively, was constitutively similar to that obtained during the breeding season (September, October. *i.e.* the extrusion-phase, see Chapter 1). However, the relative amounts of some components differed depending on the month collected. The proteins p28, OVL-50, p66 and p168 appeared to be maximally expressed during December and January, and (except OVL-50) June, whereas GDILP and p123 predominated during September and October. The expression of other components either showed no dependence on the month of collection or were expressed irregularly between individuals in a given month (p35). There were no obvious consistent differences between the six *Oa*V samples collected from individual specimens in January. Similarly, no obvious differences were apparent between the three samples obtained from individuals in September, although some proteins could not be

Table 3.1. Summary of the major characteristics of *Oa*V components

Component name	Mol. Wt. (*Da) (**kDa)	AA sequence similarities	Comments (activity, etc.)	Month of max. relative expression
1132 material	1131.9*		no A_{280}, no AAs detected following hydrolysis	N. D.
ovCNP-39A ovCNP-39B	4207.9* 4208.3*	C-type natriuretic peptide; A and B have identical AA sequences	rat uterus relaxation elevates cGMP in aortic myocytes mast cell histamine release	N. T. A.
GLP-1 GLP-2 GLP-3	5108.8* 5116.1* 4952.9*	some homology with granulins/epithelins		N. T. A. N. T. A. N. T. A.
p12/NGF	13327.5*	Nerve growth factor (NGF)	involved in activation of inward currents in sensory neurones	N. T. A.
Kunitz - like protein (KLP)	15**	some homology with kunitz - type protease inhibitor family	major protein, basic	N. D.
OVL-15	15**	lipocalin homology	shares some homology with OVL-50	N. D.
p23	23**	possible homology with insulin - like growth factor binding proteins (ILGFBPs)		N. T. A.
p28	28**			Jan, Jun
p35	35**			Sep
OVL-50	50**	lipocalin homology	shares some homology with OVL-15, acidic.	Jan
GDI-like protein (GDILP)	55**	possible homology with members of the GDP - dissociation inhibitor (GDI) family	major protein, acidic	Sep
p66	66**			Dec - Jan

Table 3.1. (cont.)

Component name	Mol. Wt. (*Da) (**kDa)	AA sequence similarities	Comments	Month of max relative expression
p123	123**		possibly related to GDILP	Sep - Oct
p168	168**			Jan, Jun
p205	205**			N. T. A.
p216	216**			N. D.

N. D. not determined
N. T. A. no trend apparent
Note: Protease activity as detected by Zymogram SDS-PAGE was associated with 3 proteins of M_r 109 kDa, 95 kDa and 84 kDa. These were not detected by protein staining, presumably due to low abundance, and are not included in this table. Hyaluronidase activity was associated with a high M_r GP-HPLC fraction containing p123 but it is unclear whether this protein is responsible for the activity (de Plater *et al.*, 1995).

visualised in the middle September sample (*i.e.* fifth lane from right of gel) as less material was available for analysis.

Most *Oa*V components could be separated effectively by RP-HPLC (μRPC, C2C18 PC3.2/3). Fig. 3.2.A shows a typical RP-HPLC separation of complete *Oa*VA annotated with the names of the major components identified. RP-HPLC was not employed routinely for the fractionation of venom proteins (>15 kDa) as extensive "ghosting" was observed on subsequent blank chromatographic runs, particularly from the high molecular weight p55/p123 material. In addition, the relatively high concentration of organic solvent required to elute the high molecular weight material increases the risk of protein denaturation and loss of activity. However, RP-HPLC was routinely employed for the separation of low molecular weight components from the venom. The C2/C18 column (PC 3.1/10), eluted with a linear CH_3CN gradient, gave very good resolution of *Oa*V peptides. Fig. 3.2B shows an example of the RP-HPLC

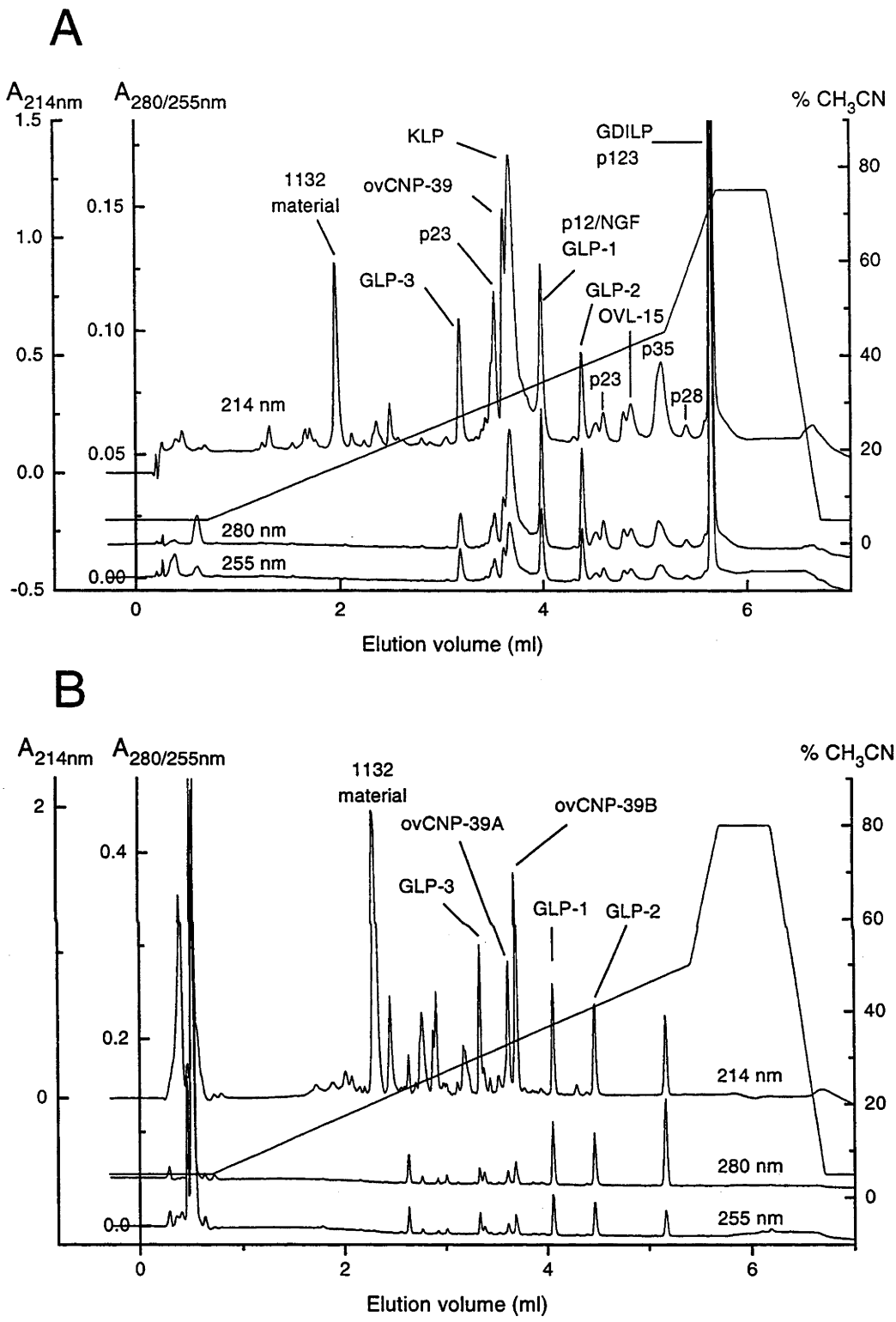


FIG.3.2. A. Reversed phase HPLC fractionation of whole *O. anatinus* venom. µRPC C2/C18 (PC3.2/3) column was used, Eluent A consisted of 0.1 % TFA / H_2O and B consisted of 0.1 % TFA / CH_3CN.). **B.** Reversed phase HPLC fractionation of a 10 kDa cutoff ultrafiltrate. A µRPC C2/C18 (PC 3.1/10) was used with the same elution conditions described for A. The chromatogram is annotated with the names of the major components identified (see Table 1).

fractionation of a 10 kDa cut-off (nominal molecular weight) ultrafiltrate. The major *Oa*V peptides could be purified to homogeneity using this two-step procedure.

1132 Da material

The major A_{214} peak resulting from RP-HPLC fractionation of the 10 kDa ultrafiltrate (Fig. 3.2B) was subjected to electrospray mass spectrometry. This component had a molecular mass of 1131.9. A minor component with a mass of 1114.9 was also present in this fraction. The material was subjected to Edman sequencing following adsorption to both PVDF and glass-fibre disc. However, no amino acids were detected. Acid hydrolysis and amino acid analysis also yielded no amino acids, although a large single peak which had apparently been derivatised by *ortho*-pthalaldehyde (OPA) was observed. The material also gave no absorbance at 280 nm (Fig. 3.2B). These results suggest that the 1132 material is not a peptide.

C-type natriuretic peptide (ovCNP-39)

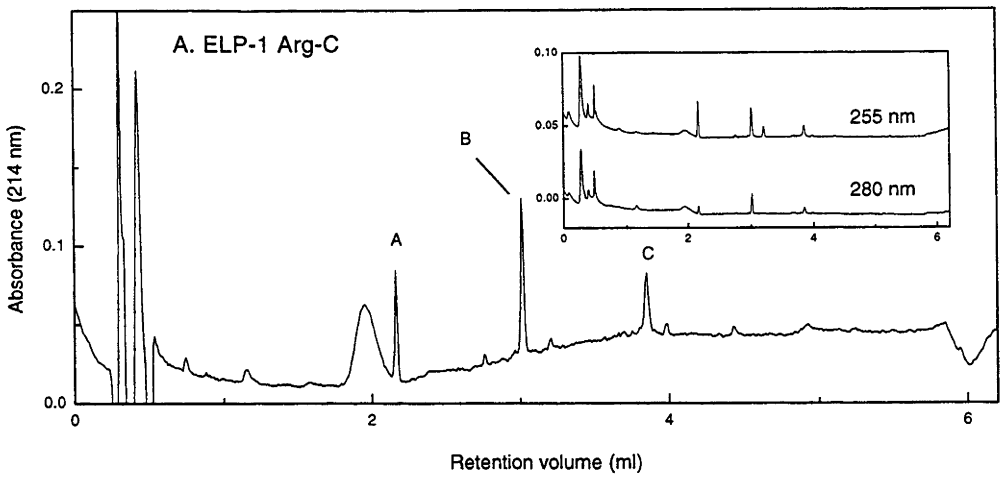
ovCNP-39 (*Oa*V C-type natriuretic peptide) was extensively studied and details of its purification and characterisation are described in Chapter 4.

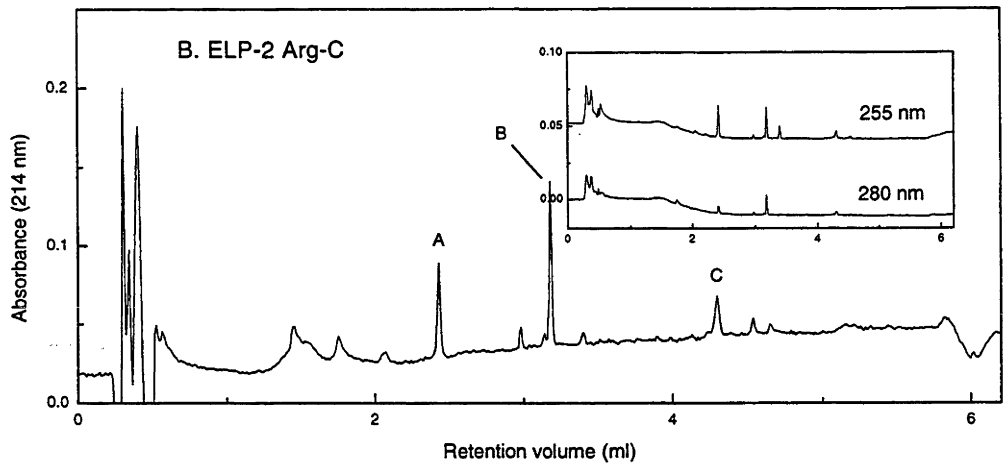
Granulin - like peptides (GLP-1, 2, 3)

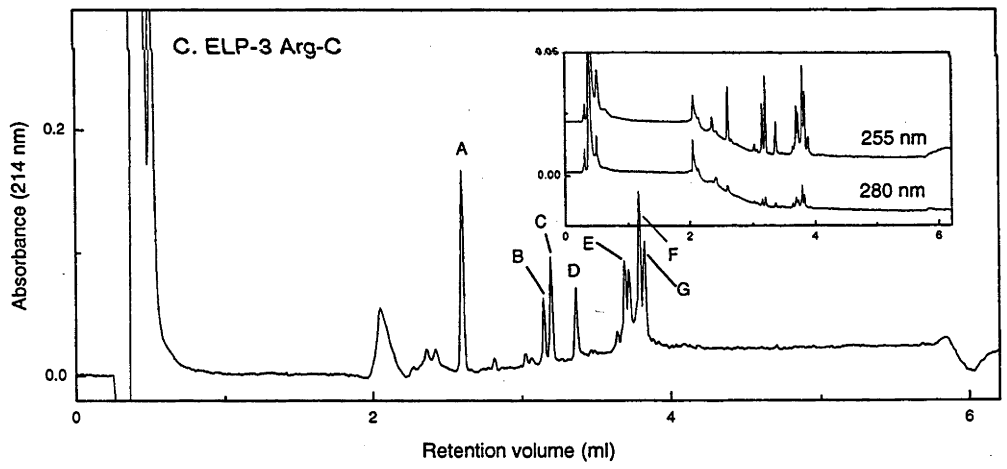
Three peptides, purified by RP-HPLC (labelled GLP-1, 2, 3 in Fig. 3.2B) were subjected to TOF-MALDI mass spectrometry, yielding molecular masses of 5108.8, 5116.1, and 4952.9, respectively. The three peptides were reduced, pyridylethylated and subjected to proteolytic digestion using endoproteinase Arg-C (GLP-1, 2 and 3, Fig 3.3A-C) and chymotrypsin (GLP-3 only, Fig. 3.3D) and the resulting fragments analysed by Edman sequencing.

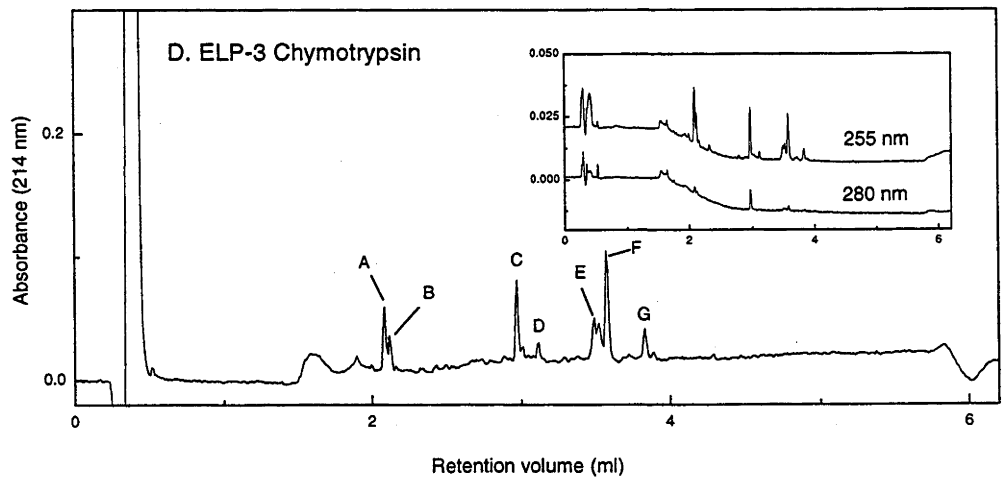
The two higher molecular weight species had very similar $A_{214}/A_{280}/A_{255}$ absorbance ratios as did their Arg-C fragments, which also had comparable retention

FIG 3.3. Reversed phase HPLC purification of proteolytic fragments generated from Endoproteinase Arg-C digestion of granulin - like peptides (GLPs) 1 (**A**), 2 (**B**) and 3 (**C**). GLP-3 was also subjected to chyotryptic digestion and the resulting fragments purified by HPLC as shown in **D**. Insets show absorbance monitored at 280 nm and 255 nm as indicated. Fragments were labelled according to their order of elution and subjected to N-terminal sequencing, the results of which are shown in Fig. 3.4.


A. ELP-1 Arg-C
Absorbance (214 nm)
Retention volume (ml)
255 nm
280 nm
A
B
C


B. ELP-2 Arg-C
Absorbance (214 nm)
Retention volume (ml)
255 nm
280 nm
A
B
C


C. ELP-3 Arg-C
Absorbance (214 nm)
Retention volume (ml)
255 nm
280 nm
A
B
C
D
E
F
G


D. ELP-3 Chymotrypsin
Absorbance (214 nm)
Retention volume (ml)
255 nm
280 nm
A
B
C
D
E
F
G

times. In contrast, the lower molecular mass species (4952.9) had relatively little absorbance at 280 nm and 255 nm (Fig. 3.2B) and gave a very different profile following Arg-C digestion (Fig. 3.3). These differences were reflected in the elucidated amino acid sequences (Fig. 3.4A). All three peptides were 42 residues in length and characterised by the presence of 6 cysteine residues at conserved positions between peptides. However, GLP (granulin-like peptide, see below)-1 and -2 appeared to have identical amino acid sequences, whereas GLP-3 is somewhat different. 33 % of the amino acid sequence of GLP-1/2 and 3 are identical. GLP-1/2 contains two Tyr and two Trp residues, compared to one Tyr in GLP-3 and this is probably responsible for the greater relative absorbance of GLP-1/2 at 280 nm. The calculated mass of GLP-1/2 is 5109.7 and that of GLP-3 is 4953.6 (assuming all Cys residues participate in disulphide bridges), and these are, with the exception of GLP-2, in close agreement with the observed masses.

A database search did not reveal any obvious similarity between these and known peptides, although some weak similarity exists with granulins, a family of cysteine-rich polypeptides of around 6 kDa. A single precursor, acogranulin, that has tandem granulin domains can potentially generate seven different forms of granulin (designated A-G, Fig. 3.4B) which are characterised by the following 12-cysteine motif, $CX_{5-6}CX_5CCX_8CCX_6XCCXDX_2HCCPX_4CX_{5-6}C$ (Bhandari *et al.*, 1993). Thus, the *Oa*V peptides contain half the number of cysteine residues as other granulins but the spacing between them is conserved and the CC motif near the carboxy-terminus which is common to all granulins, is also conserved. The OaV peptides have therefore been given the tentative name, granulin-like peptides or GLPs (Fig. 3.4B). GLP-1, 2 and 3 all share 19 % sequence identity with rat granulin A.

FIG. 3.4. A. The elucidated amino acid sequences of GLP-1 and 2 determined from direct N-terminal sequencing and from proteolytic fragments (Arg-C). Note that the sequences are identical **B.** The elucidated sequence of GLP-3, showing overlap of Arg-C and chymotryptic fragments (see Fig. 3.3) used to determine the sequence. N-term refers to sequence obtained directly from the N-terminus; ArgCA1 and 2 are two sequences obtained from the single peak A (Fig. 3.3C). **C.** Sequence alignments showing overlap between GLP-1/2, GLP-3 and rat and carp granulins and the locust brain granulin, PMP-D1.

Nerve growth factor (p12/NGF)

p12/NGF (nerve growth factor) was extensively studied and its purification and characterisation is not dealt with here but described in detail in Chapter 6.

Kunitz-type protease inhibitor-like protein (KLP)

Whole venom was fractionated by cation-exchange HPLC using a MonoS column and a linear 0-1 M NaCl gradient in 20 mM HEPES buffer, pH 7.5. The major component retained by the column was a 15 kDa protein which eluted at a high NaCl concentration (peak labelled KLP, Fig. 3.5A). The fraction was desalted by ultrafiltration (10 kDa NMWL). Dilution of the fraction was required prior to ultrafiltration as the material tended to precipitate as it became more concentrated. The protein was further purified by RP-HPLC on a C2/C18 (PC 3.2/3) column and eluted with CH_3CN in 0.1 % TFA (Fig. 3.5B). The protein was thus purified to homogeneity as judged by SDS-PAGE (Fig. 3.5A, inset). It was reduced, pyridylethylated and subjected to chymotryptic and endoproteinase Arg-C digestion (Fig. 3.6A and B, respectively) and some of the resulting peptide fragments were subjected to Edman sequencing. The intact protein was also subjected to direct N-terminal Edman sequencing. The resulting amino acid sequences are given in Fig. 3.6. Fragments of this protein were found to be homologous with members of the pancreatic trypsin inhibitor, or Kunitz, family and the protein has therefore been designated Kunitz-type protease inhibitor-like protein (KLP). When the fragments are aligned in such a way as to maximise amino acid overlap with members of the Kunitz family (Fig. 3.7), KLP conforms to the consensus pattern of $FX_3GCX_6FX_6C$ (Laskowski and Kato, 1980; Salier, 1990; Ikeo *et al.*, 1992). In addition, the active-site domain of Kunitz-type inhibitors, consisting of a GXCK motif (Kondo *et al.*, 1982; Salier, 1990; Ikeo *et al.*, 1992), is conserved in KLP (residues 25-28, Fig. 3.7). Bovine plasma trypsin inhibitor (BPTI) and cow colostrum

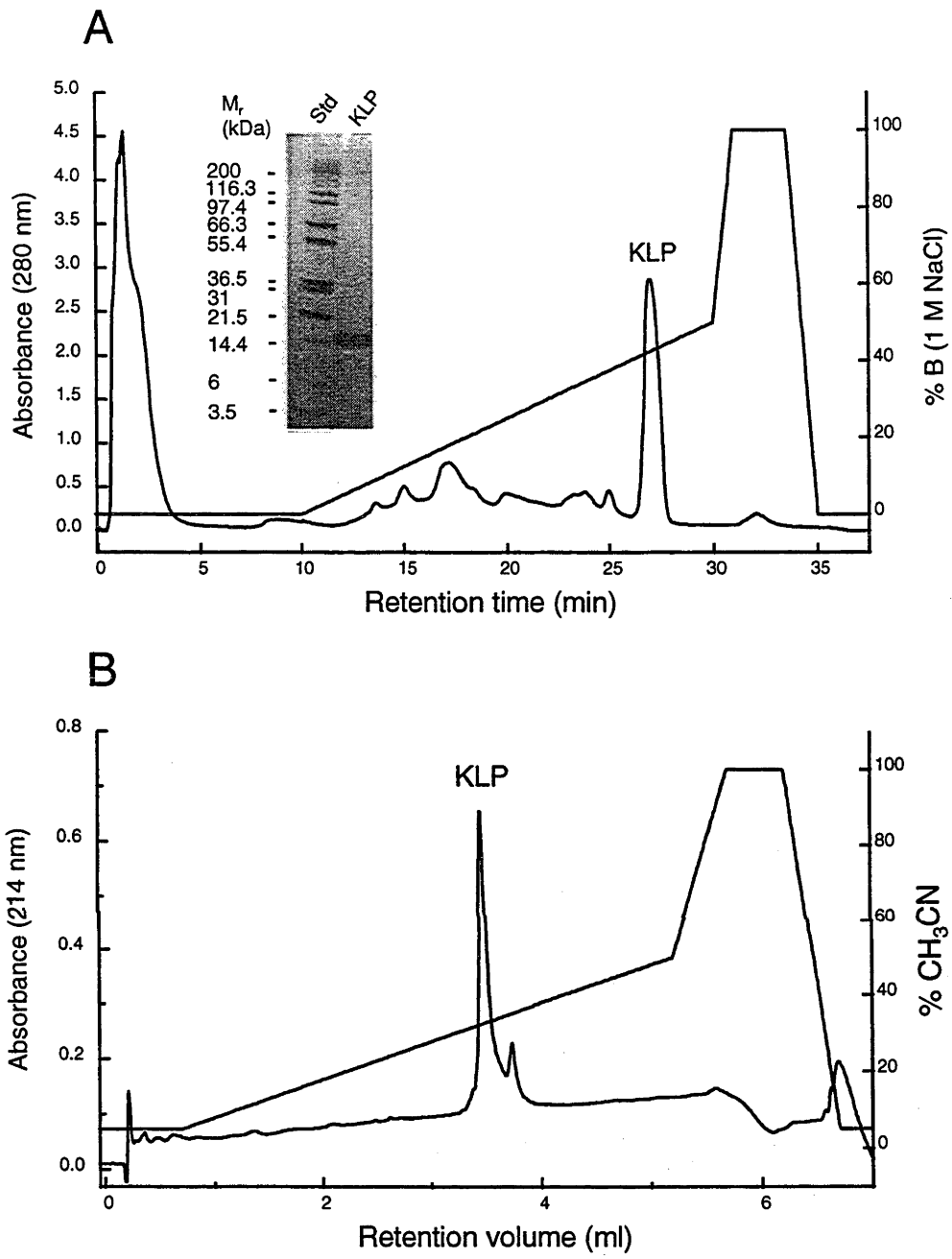
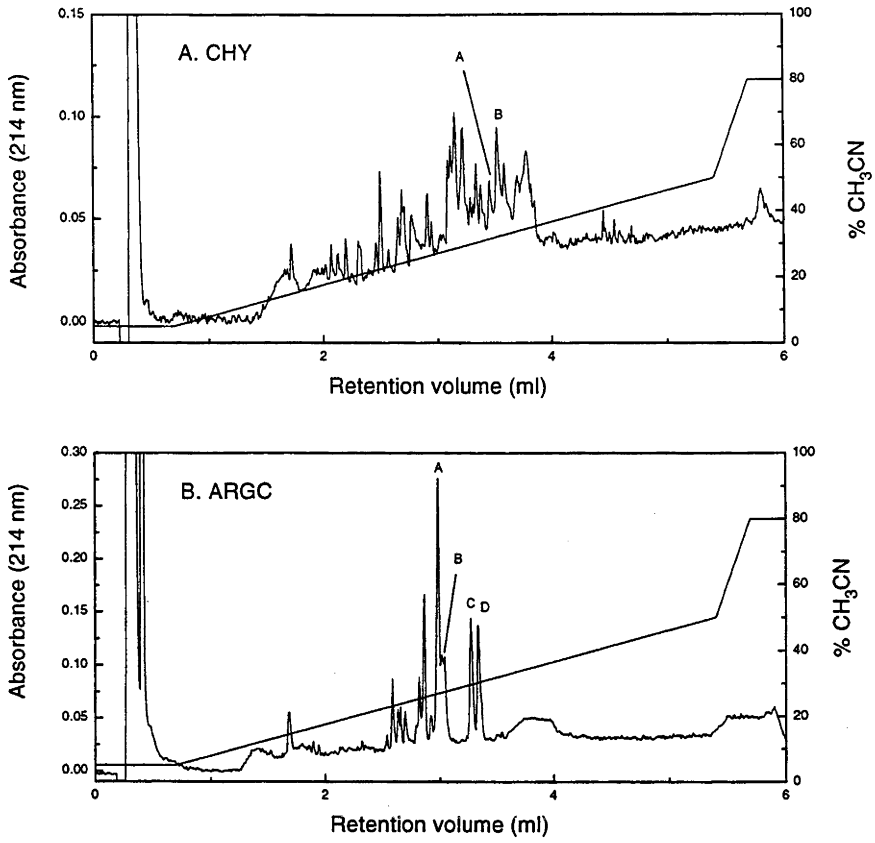


Fig. 3.5. Purification of the 15 kDa KLP from whole *O. anatinus* venom using **A.** cation - exchange (MonoS, 0-1 M NaCl gradient in 50 mM HEPES, pH 7.5) and **B.** Reversed phase HPLC (µRPC C2/C18, PC 3.2/3, 0-100 % CH_3CN in 0.1 % TFA). The inset in **A.** shows the Tris-Tricine SDS-PAGE analysis of purified KLP.



C

Peak	Sequence
CHY-A	C Q L P P L K G Q C
CHY-B	C Q L P P L K G Q C P S M
ARGC-A	K G P C K E K H H Q Y Y F N M A T R
ARGC-B	F Q T K E E C Q M T C F P V G A
ARGC-C	F G S H E K C L A T C G I S G I P P V C Q L P R
ARGC-D	C V P F F Y N G C G G
N-term.	R K S V T E L C Q L P P L K G Q C P S M M E R

FIG. 3.6. Reversed phase HPLC purification of proteolytic fragments of KLP generated from chymotryptic (**A**) and endoproteinase Arg-C (**B**) digestion. Several peptide fragments were subjected to Edman sequencing and the resulting amino acid sequences are given in **C**. The protein was subjected to direct N-terminal sequencing, the results of which are also shown in **C**.

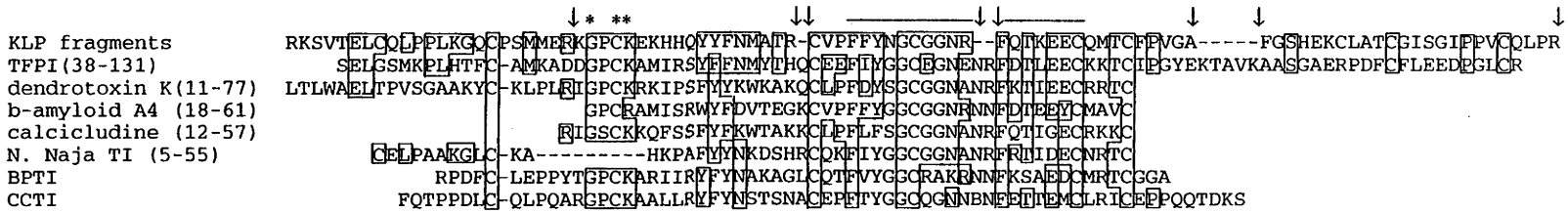


Fig. 3.7. Comparison of the amino acid sequence of KLP with those of several members of the Kunitz - type protease inhibitor family. Arrows indicate the beginning and end of each region of elucidated sequence and asterisks indicate the conserved residues of the active site domain of Kunitz - type protease inhibitors. The horizontal bar above the KLP sequence indicates the Kunitz - type inhibitor consensus pattern, ie. $FX_3GCX_6FX_6C$. Note that KLP conforms to this consensus sequence. Abbreviations are as follows. TFPI, tissue factor pathway inhibitor (rat); BPTI, bovine plasma trypsin inhibitor; CCTI, cow colostrum trypsin inhibitor. Dendrotoxin K is from *Dendroaspis polylepsis*, amyloid A4 is from mouse and calcicludine is from *Dendroaspis angusticeps*.

trypsin inhibitor (CCTI) are considered prototype members of the Kunitz family although there are many other members. The amino acid sequences of several of these are compared in Fig. 3.7.

15 kDa OaV lipocalin (OVL15)

Gel permeation HPLC (Superose 12) of *Oa*VA revealed a 15 kDa protein (Fig 3.8A) which differed from KLP in that it eluted differently on RP-HPLC (Fig. 3.8B) and was not retained by the MonoS column. The peptide was subjected to N-terminal Edman amino acid sequencing and 25 residues of sequence were obtained. The protein appears to be a member of the lipocalin family (Fig. 3.9), a group of proteins which play a role in the binding and transport of small hydrophobic molecules such as steroids, retinoids and lipids. Members of this family share a common tertiary structure and three conserved stretches of sequence (Flower *et al.*, 1991; Flower *et al.*, 1993). The first of these is common to all lipocalins and is considered a "signature" pattern. The stretch is [DENG]-X-[DENQGSTARK]-$X_{0,2}$-[DENQARK]-[LIVFY]-XXX-G-X-W-[FYWLRH]-X-[LIVMTA], where any of the residues enclosed in [] may be present at that position. The N-terminal sequence of the 15 kDa protein (designated OVL15 for *Ornithorhynchus* venom lipocalin, 15 kDa) conforms to this signature pattern and has a number of additional residues in common with core lipocalins (Fig. 3.9). The N-terminal sequence of OVL-15 shares 56 % identity with the overlapping 25 N-terminal residues of the other *Oa*V lipocalin, OVL-50 (see below).

p23

A 23 kDa protein, designated p23, was purified from *Oa*V using a combination of cation exchange (MonoS, Fig. 3.10A) and RP-HPLC (C2/C18, PC3.1/10) (Fig. 3.10B). A broad elution profile from Mono S was obtained, p23 being present in several fractions. It was purified to near-homogeneity as judged by SDS-PAGE (Fig 3.10C)

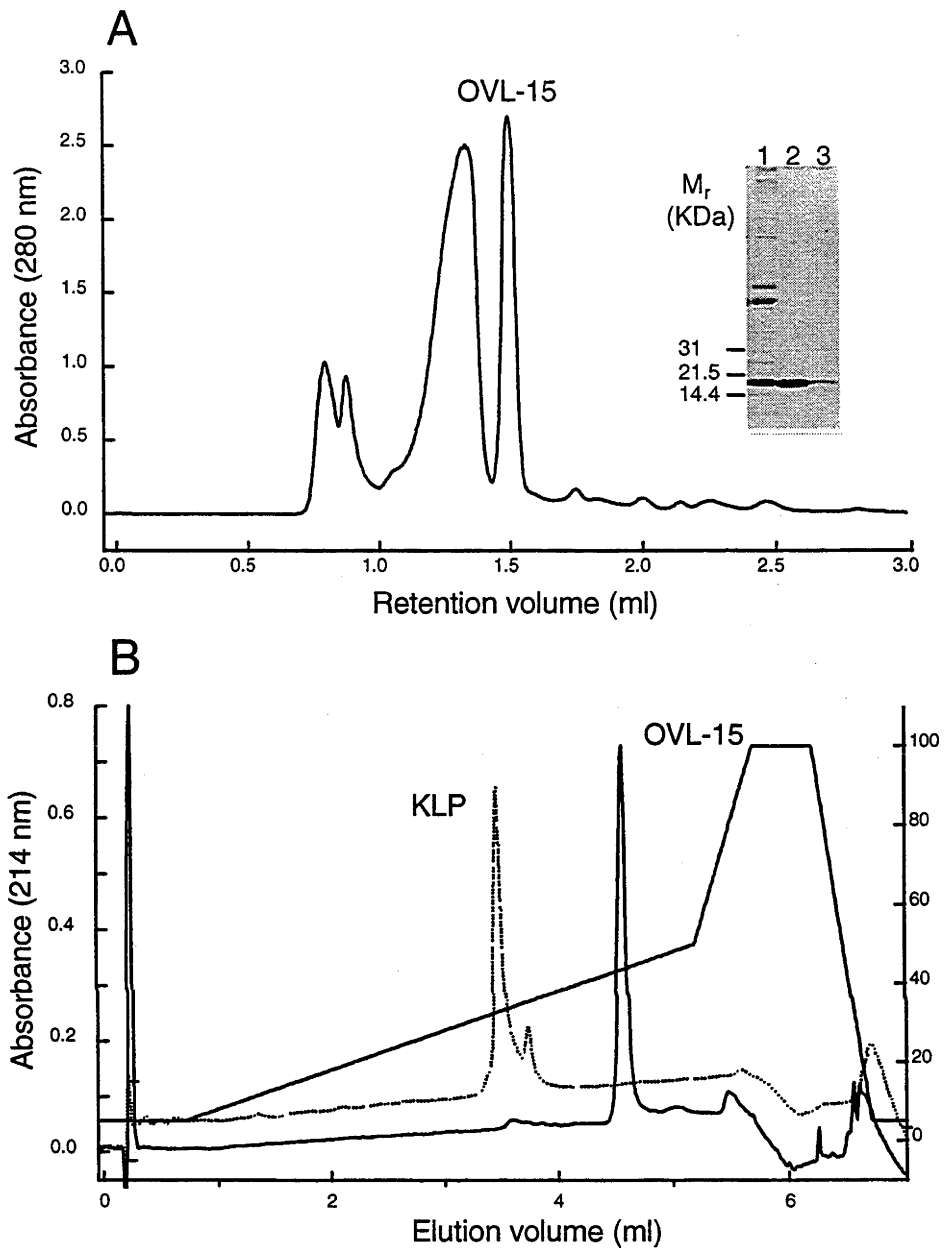


FIG. 3.8. Purification of the 15 kDa OVL-15 from whole *O. anatinus* venom using **A.** Gel permeation HPLC (Superose 12, 50 mM MES pH 6.1) and **B.** Reversed phase HPLC (mRPC C2/C18, 3.2/3.0, 0-100% CH3CN in 0.1% TFA). Note the difference in retention time between KLP (dotted line) and OVL-15 (solid line). The inset in **A** shows the Tris-Glycine SDS-PAGE analysis of purified OVL-15. Lane 1, OaV; Lane 2, OVL-15 ex GP-HPLC; Lane 3, OVL-15 ex RP-HPLC.

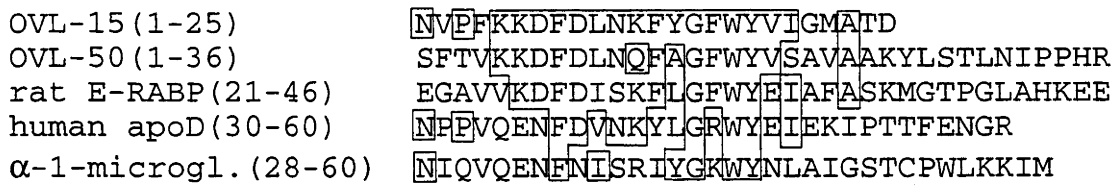


FIG. 3.9. Comparison of the elucidated N-terminal amino acid sequence of OVL-15 with those of OVL-50 and members of the lipocalin family. Abbreviations are as follows. E-RABP, epididymal-retinoic acid binding protein precursor; apoD, apolipoprotein D; α-1-microgl., α-1-microglobulin.

and reduced, pyridylethylated and subjected to N-terminal amino acid sequencing. Ten residues of sequence were obtained (Fig. 3.10D). The sequence shows some similarity to members of the insulin-like growth factor binding protein (IGFBP) family, *e.g.* 40 % identity with mouse connective tissue growth factor (FISP-12). However, as only 10 residues of sequence were obtained, the significance of this overlap is difficult to determine. In addition, the consensus sequence for this protein family, GC[GS]CCX$_2$CAX$_6$C, (Bradham *et al.*, 1991; Joliot *et al.*, 1992) would occur downstream from the elucidated sequence and, therefore, could not be used to confirm the structural relationship between p23 and members of the IGFBP family.

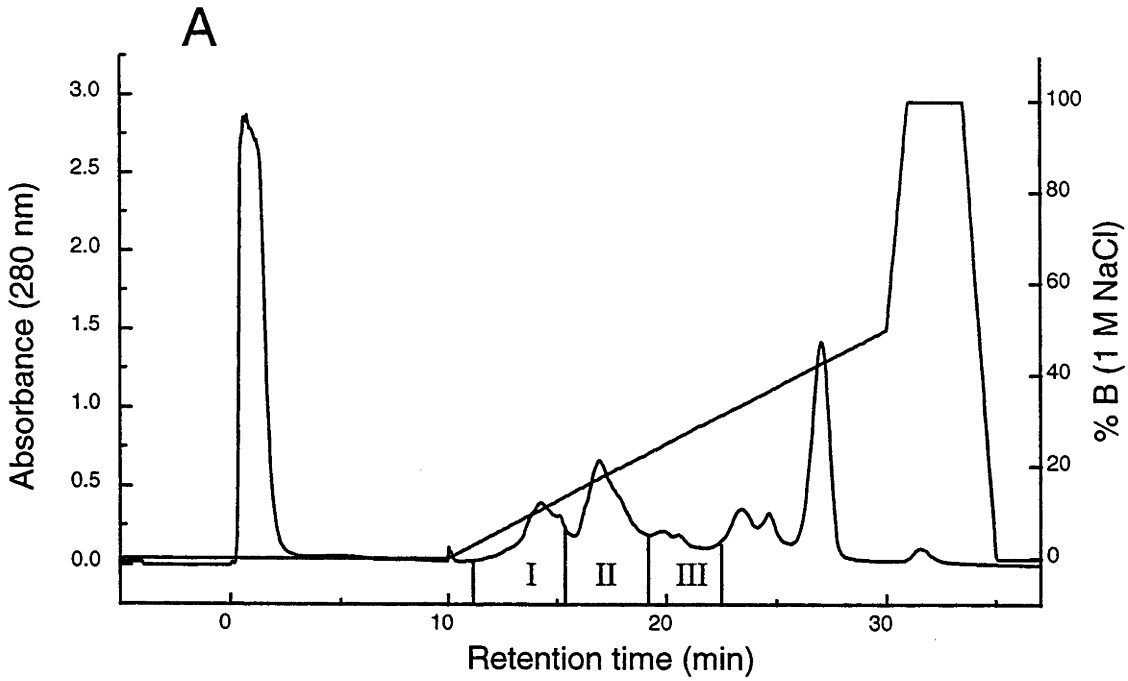
50 kDa OaV lipocalin (OVL-50)

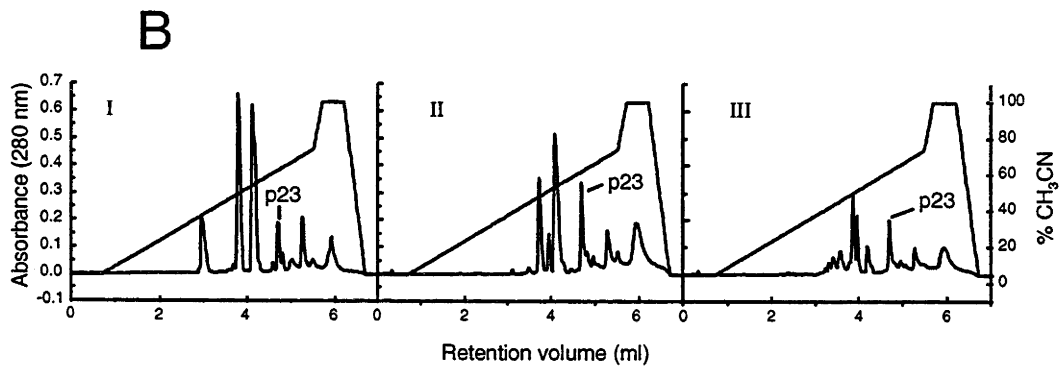
A 50 kDa protein was partially purified from the venom by anion-exchange HPLC on MonoS, in 20 mM bis-Tris, pH 6.5, using a linear gradient of 0 - 0.5 M NaCl (Fig. 3.11A, fraction D). This material was analysed by SDS-PAGE and the band, running at an apparent M_r of 50 kDa, was excised and divided in half. Half was subjected to direct N-terminal Edman sequencing and half to in-gel tryptic digestion. RP-HPLC analysis of the resulting tryptic fragments is shown in Fig. 3.12. Twelve A_{214} peaks were subjected to Edman sequencing and the resulting sequences are also shown in Fig. 3.12. The protein, like OVL-15, appears to be a member of the lipocalin family from its N-terminal sequence (see alignment in Fig. 3.9) and has thus been named OVL-50. However, no consistent homologies were observed between the other OVL-50 tryptic fragment sequences and known proteins.

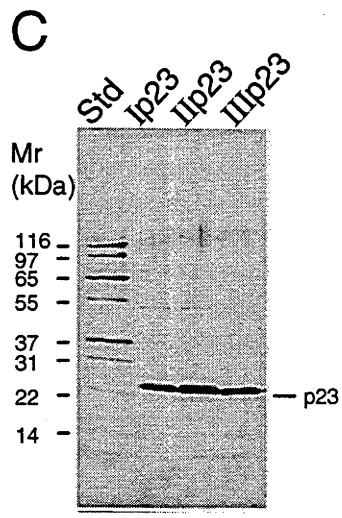
55 kDa guanosine diphosphate dissociation inhibitor-like protein (GDILP)

The 55 kDa protein, which was the most abundant protein in most *Oa*V samples, was excised from an SDS-PAGE gel (see Fig. 3.1) and subjected to direct N-terminal Edman sequencing and in-gel tryptic digestion. The resulting tryptic fragments were

FIG 3.10. Purification of p23 from *O. anatinus* venom by **A.** Cation - exchange HPLC (MonoS, 0 - 1 M NaCl gradient in 50 mM HEPES, pH 7.4) and **B.** Reversed phase HPLC (μRPC C2/C18, PC 3.2/10, 0 - 100% CH_3CH in 0.1 % TFA). Note that p23 was present in each of cation - exchange fractions I, II and II. **C.** SDS-PAGE analysis of purified p23, *ex* RP-HPLC, corresponding to cation exchange HPLC fractions as indicated. Std, molecular weight protein standards **D.** N-terminal amino acid sequence of p23 and its comparison with those of members of the insulin-like growth factor binding protein family (precursor forms). Numbers at the end of each sequence correspond to the position number of the final amino acid residue of that sequence. Abbreviations are CTGF, connective tissue growth factor; CE10, CEF-10; and CYR6, cyr61.







D

N-term: ALGQDCEEQQ

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1 p23 N-TERM          ALGQDCEEQQ                10
2 CTGF_MOUSE GCCRVCAKQLGELCTERDPCDPHKGLFCDF 83
3 CTGF_HUMAN GCCRVCAKQLGELCTERDPCDPHKGLFCDF 84
4 NOV_COTJA  GCCLVCARQRGESCSPLLPCDESGGLYCDR 89
5 CE10_CHICK GCCKVCAKQLNEDCSRTQPCDHTKGLECNF 80
6 CYR6_MOUSE GCCKVCAKQLNEDCSKTQPCDHTKGLECNF 80
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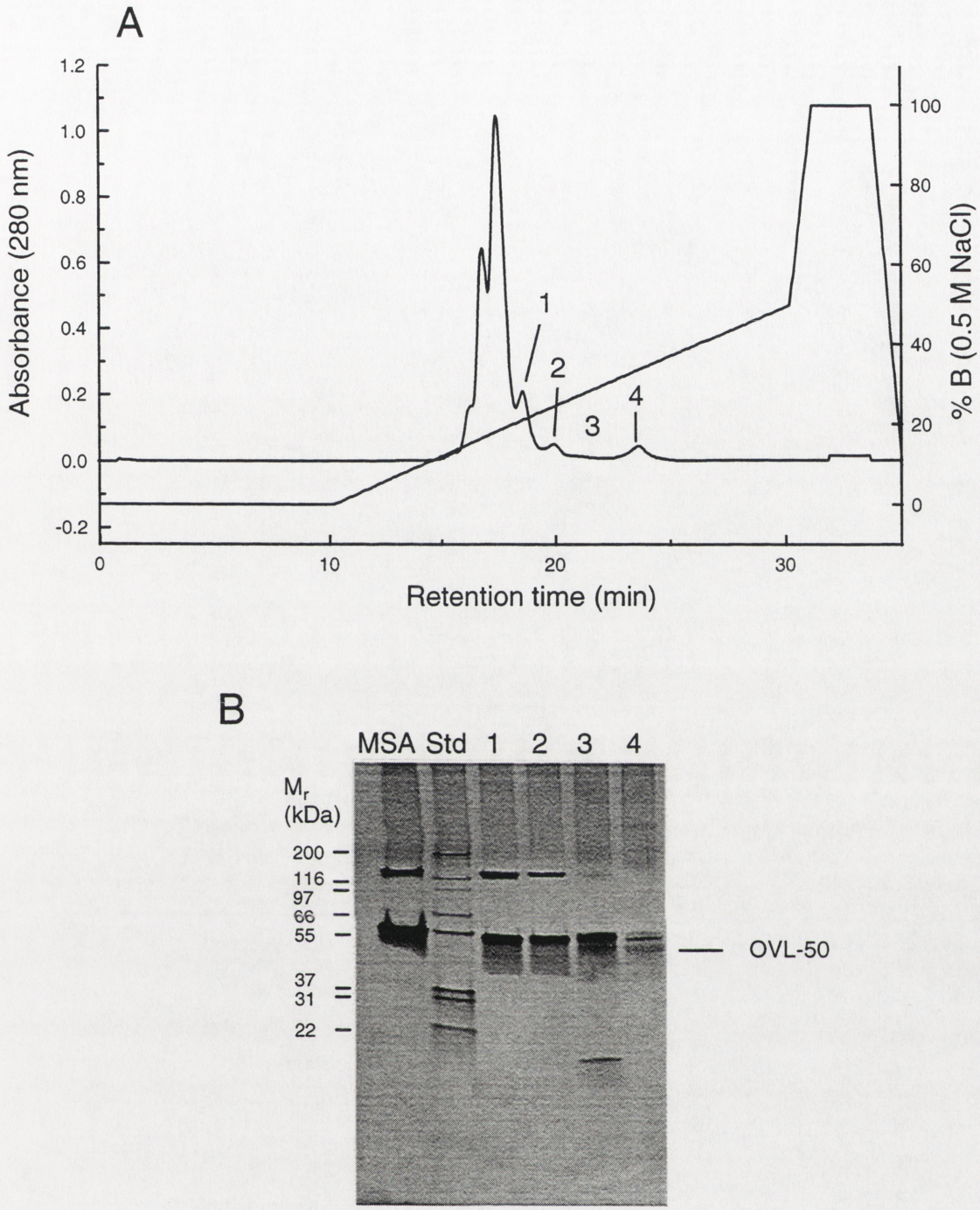
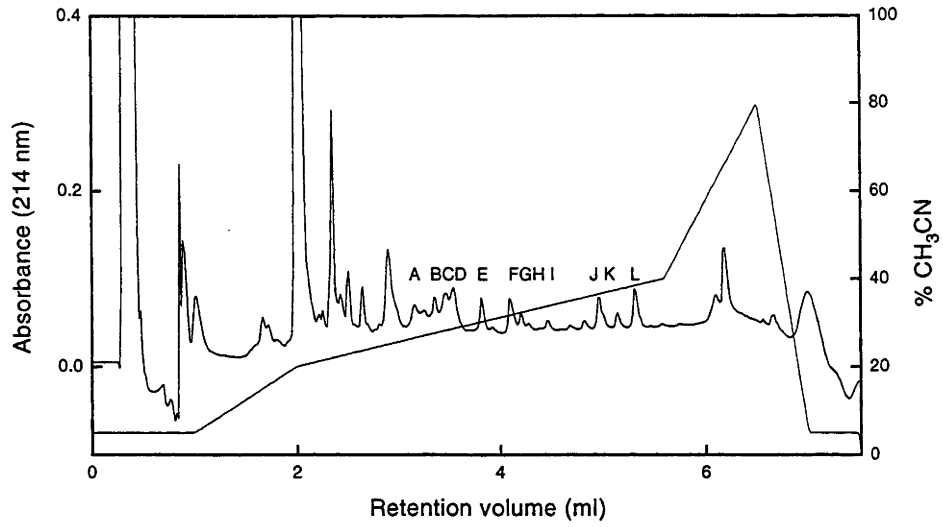


FIG. 3.11. A. Anion - exchange HPLC (MonoQ, 20 mM bis-Tris, pH 6.5, 0 - 0.5 M NaCl gradient) partial purification of the 50 kDa protein, OVL-50 from a MonoS flow-through fraction (see fig. 3.10). **B.** SDS-PAGE analysis of MonoQ fractions. MSA, MonoS flow - through fraction. Std, molecular weight protein standards.



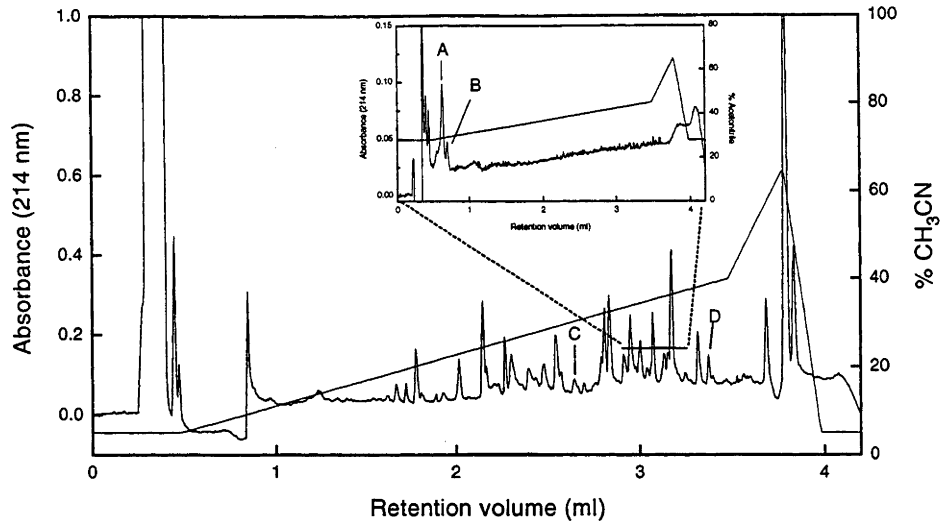
Peak	Sequence
A	T G G V L V T P H V G G V R F
B	F A F V L T S G K
C	K A F V L V F G V I Q
D	L I K Q D Y F C V T E S I X P
E	F Q I N P A I S L T V V D T D
F	D A E G D F N F L M A Y G R
G	F A E G D F N F L M A Y G R
H	F C L A L Y N E G I P H T Q T
I	F A E G D F N F L M A Y G R
J	K D F D L N
K	K D F D L N Q F A G F
L	D F D L N Q F A G F
N-term.	S F T V K K D F D L N Q F A G F W Y V S A V
	A A K Y L S T L N I P P H R

FIG 3.12. Reversed phase HPLC purification of proteolytic fragments of OVL-50 generated from in-gel tryptic digestion. Several peptide fragments were subjected to Edman sequencing and the resulting amino acid sequences are shown below the chromatogram as is the elucidated N-terminal sequence. A comparison of this sequence with those of OVL-15 and other lipocalins is shown in Fig. 3.9.

purified by RP-HPLC using a Sephasil C18 column (Fig. 3.13). The purified peptides were subjected to Edman sequencing and the sequences obtained are shown in Fig 3.13. No conclusive homology to known proteins is apparent, although there is limited similarity to members of the guanosine diphosphate dissociation inhibitor (GDI) superfamily, including Rab-GDI and the choroideremia gene product, CHM alternatively known as Rab-escort protein (REP) (Waldherr *et al.*, 1993; Wu *et al.*, 1996). Sequence alignments between these GDI family members and the 55 kDa protein from *Oa*V are shown in Fig. 3.14. Members of the superfamily show several highly conserved regions, including the $DVX_3GTGX_2EX_2L$ motif (Sequence Conserved Region, SCR, 1A) beginning at residue 6 (Wu *et al.*, 1996). The 55 kDa protein, tentatively named GDILP (GDI-like protein) possesses the conserved DV diad at residue 6 (see Fig. 3.14.) (Wu *et al.*, 1996). N-terminal sequence beyond residue 10 was not obtained and therefore it was not possible to determine whether the remainder of the SCR1A motif was conserved. No other sequences were obtained within other SCR sites to confirm the GDI homology.

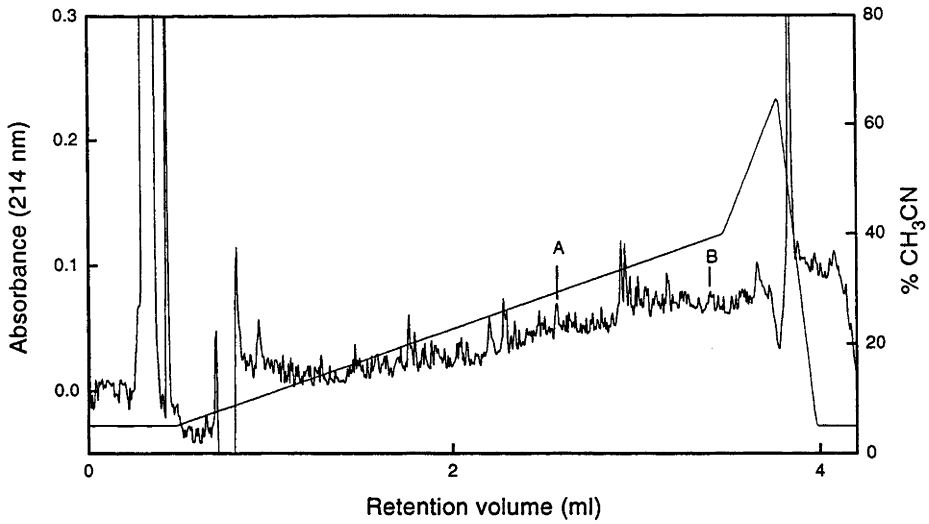
p123

A protein band corresponding to 123 kDa from *Oa*V (p123) was excised from an SDS-PAGE gel and subjected to in-gel tryptic digestion. The resulting peptide fragments were purified as described for GDILP (Fig. 3.15). Two resulting peptide fragments were subjected to Edman sequencing and the sequences obtained are given in Fig. 3.15. Peptide A(1) shares 67 % identity with peptide C from GDILP while peptide B was truncated but otherwise identical to peptide D from GDILP. Peptide A(2) did not show any overlap with peptides from GDILP and database searching revealed no obvious homologies.



Peak	Sequence
A	F N R P N L M G V F E
B	S Y D V N V M N
C	T L P L A Y P Q I
D	K V N M D F L D A L K
N-term.	D P E E D V G P A

FIG 3.13. Reversed phase HPLC purification of proteolytic fragments of the 55 kDa protein, GDILP, generated from in-gel tryptic digestion. The inset shows the re-purification of fractions underlying the horizontal bar, using a shallower gradient. Several peptide fragments were subjected to Edman sequencing and the resulting amino acid sequences are shown below the chromatogram. 9 residues of N-terminal amino acid sequence were also elucidated and this is shown below the proteolytic fragment sequences.



Peak	Sequence
A	T N P L A L P A I (1)
	F I R M Y L R (2)
B	V N M D F L D A L

FIG 3.15. Reversed phase HPLC purification of proteolytic fragments of the 123 kDa protein (p123), from *O. anatinus* venom, generated from in-gel tryptic digestion. Several peptide fragments were subjected to Edman sequencing and the resulting amino acid sequences are shown below the chromatogram. Peak A gave rise to both a major (1) and minor (2) sequence.

Discussion

A total of 19 components were identified in *Oa*V. Several of these were purified and either fully or partially sequenced and in some cases demonstrated to belong or be related to specific protein/polypeptide families. Some proteins exhibited a difference in their relative level of expression at different times of the year, while the remainder showed no apparent trend.

Temple-Smith (1973) found that high molecular weight proteins varied irregularly depending on the time of year, whereas "postalbumins" predominated in material taken from extrusion-phase glands, and "prealbumin" proteins predominated in material taken from regressing and quiescent crural glands. A direct comparison between this and data obtained in the present study is difficult as the former only classified proteins into molecular weight ranges. However, a comparison of the relative proportions of proteins from the electrophoretic analysis of Temple-Smith (1973) with that of the present study suggests that the major "post-albumin" component is actually the 55 kDa GDILP. This being the case, the increased expression of GDILP in September is consistent with the increase in "post-albumins" observed by Temple-Smith. However, p66, another "post-albumin", appeared to be maximally expressed in December and January when crural glands are in regression. Among the high molecular weight proteins, p123 expression predominates in extrusion-phase material while p168 predominates in regression- and elaboration-phase material (see Fig. 3.1C).

As the SDS-PAGE comparisons of relative *Oa*V protein expression were qualitative, only major trends were easily discernible. In addition, material from only 1 elaboration-phase, 4 extrusion-phase and 6 regression-phase individuals were compared and a more exhaustive study may reveal more inter-individual variation within a given collection time. Nevertheless, the results do suggest a seasonal variation in some venom components and this is consistent with observed seasonal changes in

the secretory activity of crural glands (Temple-Smith, 1973). As the crural gland actively secretes material only during the breeding season, material taken outside this time has presumably undergone a period of storage. The selective reduction in the apparent concentrations of p123 and GDILP outside the breeding season suggests that these proteins may be susceptible substrates for proteolytic enzymes in *Oa*V (Temple-Smith, 1973; de Plater, Hons thesis, ANU, 1993).

Apart from the C-type natriuretic peptide and p12/NGF, none of the components of *Oa*V could be unequivocally identified based on their amino acid sequences. However, in several cases, homology was found between *Oa*V components and particular protein families or superfamilies. For the *Oa*V lipocalins (OVL-15 and OVL-50) and Kunitz-type protease inhibitor-like protein (KLP), "signature" domains which are universally conserved between members of these families were found in the corresponding *Oa*V proteins. Thus, it is possible to speculate upon their function based on the activities of other family members with which they share homology. Each of the major components identified in *Oa*V and characterised are discussed in the following sections.

Non-peptide/protein material

*Oa*V contains large quantities of a low molecular weight material (mol. wt. 1131.9), designated 1132 material, which was refractory to Edman sequencing and did not yield amino acids following acid hydrolysis. These results suggest the 1132 material may not be a peptide. Although approximately 90 % of the dry weight of most venoms consists of protein material, many non-protein components, including metal ions, inorganic anions, lipids, nucleosides, carbohydrates and amines, are also present (Bieber, 1979). The molecular weight of the 1132 material and its reaction with OPA suggests it may be peptide-like, although refractory to Edman sequencing and resistant

to acid hydrolysis. Acylpolyamines may behave in such a way. These compounds, which block and modulate several types of ion channel, are found both endogenously and in arthropod venoms (Williams, 1997). Whether or not the 1132 material is a polyamine may be established by NMR and further mass spectrometric analyses.

OaV peptides

Three related, 42 residue peptides have been purified from *Oa*V and sequenced. They have been tentatively designated GLP (granulin-like peptide) -1, 2 and 3. Although the homology between GLPs and granulins is limited, the positions of each of the 6 GLP cysteine residues align with cysteine residues of granulins. This suggests they may share a similar pattern of disulphide connectivity and topology, although granulins contain twice as many cysteines in total as GLPs. Like GLP, several forms of granulin have been identified, where a single precursor, acogranulin, which has tandem granulin domains, gives rise to the multiple forms. The GLPs are also similar in size to granulins, typically around 6 kDa. Granulins play a role in the regulation of epithelial proliferation (Bhandari *et al.*, 1993) and their presence in leukocytes and inflammatory exudates suggests possible roles in inflammation and wound repair (Bhandari *et al.*, 1992). Other members of the granulin family include an equine neutrophil antimicrobial peptide, eNAP-1 (Couto *et al.*,1992) and a locust brain peptide (PMP-D1) of unknown function (Nakakura *et al.*, 1992), the sequences of which are given in Fig. 3.4C. Although granulin-like peptides have not been previously described in a venom, granulins contain a subdomain comparable to the N-terminal subdomain of small alpha-toxins from scorpion venoms, which slow Na^+ channel inactivation (Hrabal *et al.*, 1996; Lin and Nussinov, 1995).

Kunitz-type protease inhibitor-like protein (KLP)

A 15 kDa basic protein has been purified from the venom and partially structurally characterised. It is a member of the Kunitz-type protease inhibitor family, and has therefore been named KLP (Kunitz-type protease inhibitor-like protein). A number of snake venom basic protease inhibitors, which include the dendrotoxins, a group of neuronal K^+ channel blockers, are also members of the Kunitz family and share homology with KLP (Harvey, 1997). Another member of this family is tissue factor pathway inhibitor (TFPI), an endogenous anticoagulant which acts by inhibiting activated factor X and producing a feedback inhibition of the factor VIIa/tissue factor complex (Broze, 1995), whose sequence is compared with that of KLP in Fig. 3.7. Other homologous members of the Kunitz family include domains found in Alzheimer's amyloid beta-protein (Ikeo *et al.*, 1992) and calcicludine, a blocker of high-threshold Ca^{2+} channels from *Dendroaspis angusticeps* venom (Schweitz *et al.*, 1994). β-bungarotoxins from *Bungarus multicinctus* venom also have a Kunitz-like structure but lack a reactive-site Lys or Arg (see above) and therefore have no protease inhibitor activity (Kondo *et al.*, 1982). Whether KLP functions as a protease inhibitor in *Oa*V, or has evolved a specific function is unknown at this stage, although preliminary results (de Plater, unpublished) suggest that KLP, unlike BPTI (the "prototype" Kunitz-type protease inhibitor), does not inhibit trypsin. This is intriguing as the active site domain, consisting of a GXCK motif is conserved in KLP (residues 25-28).

Lipocalins

Two lipocalins, a 50 kDa (OVL-50) and a 15 kDa (OVL-15) form (OVL: *Ornithorhynchus* venom lipocalin), have been identified in *Oa*V and partially sequenced. Both OVL-50 and OVL-15 clearly contain the lipocalin "signature" pattern

and share a relatively high degree of homology with core members of the lipocalin family. Lipocalins are generally involved in the binding and transport of small hydrophobic molecules. Examples include epididymal retinoic acid binding protein (E-RABP), an androgen-dependent protein found in the lumen of the epididymis which is required for sperm maturation (Newcomer, 1993); apolipoprotein D (apoD), which is implicated in the transport of several small hydrophobic molecules including sterols and steroid hormones and which may play a key role in cholesterol homeostasis (Suresh *et al.*, 1998); and α-1-microglobulin whose function is unclear, but interestingly, is derived from the same precursor protein as bikunin, a Kunitz-type protease inhibitor (Chan and Salier, 1993). Lipocalins have not been previously identified in a venom and speculation on the role of the OVL proteins is difficult although at least two lipocalins, human NGAL (Neutrophil gelatinase-associated lipocalin) and mouse 24p3 are acute-phase proteins[10] which may play important roles in inflammation (Liu and Nilsen-Hamilton, 1995; Cowland and Borregaard, 1997).

Other proteins

It was not possible to establish the significance of the N-terminal sequence similarity between the 23 kDa protein and members of the insulin-like growth factor binding protein (IGFBP) family, as only 10 residues were obtained and the consensus sequence for this family lies downstream from the elucidated sequence. Members of the IGFBP family include the mouse protein, cyr61 and its chicken homologue, CEF-10 which are growth factor-inducible immediate-early gene products thought to act cooperatively with growth factor activities (Bradham *et al.*, 1991; Kireeva *et al.*, 1998). Connective tissue growth factor (CTGF), a mitogenic peptide that binds heparin and is secreted by

[10] Acute-phase proteins are hepatocyte-derived serum proteins produced during the early stages of an inflammatory response (Kuby, 1992)

fibroblasts after activation with transforming growth factor beta (TGF-β) (Grotendorst, 1997), is also a member of the IGFBP family. No members of this family appear to have been found previously in a venom.

The most abundant protein in the majority of *Oa*V samples was a 55 kDa protein which was partially sequenced. Although only limited sequence data were obtained, the protein appears to show some homology to members of the GDP-dissociation inhibitor (GDI) superfamily, including Rab-GDI, which plays an important role in the recycling of GTPases (Rab proteins) involved in vesicular membrane transport (Wu *et al.*, 1996). The significance of the similarity between the 55 kDa protein, which has tentatively been designated GDILP (GDI-like protein), and GDI proteins is difficult to determine as the N-terminal region contained the only elucidated sequence which fell within a conserved GDI region. A 123 kDa *Oa*V protein, p123, shares some homology with GDILP, suggesting that the two proteins are related and that p123 may in fact be a precursor of GDILP.

General comments

Structural similarity in proteins may reflect evolution from a common ancestral protein with a divergence of function. For example, snake venom cardiotoxins and crotamine share homology with phospholipases A and ribonuclease respectively and probably share a common ancestral protein, although their activities and functions have clearly diverged (Strydom, 1979). In addition, animal toxins with unrelated biological functions very often possess a similar architecture. This may occur because certain motifs, for example the snake toxin fold[11], undergo a "natural engineering", which may be associated with an accelerated rate of evolution (Ohno *et al.*, 1998). In contrast, the

[11] the snake toxin fold is a common motif shared by phospholipases A_2 from Viperid and three-fingered toxins from Elapid and Hydrophiid snake venoms

core structure of the protein, which may not be subject to the same evolutionary pressure, is conserved. Therefore speculation on the function of *Oa*V proteins, based on their amino acid sequence homology must be viewed with due caution.

It is clear that many of the proteins and peptides in *Oa*V, with the exception of NGF and ovCNP-39, are unique and although often showed some similarity to other proteins, these proteins have not previously been identified in a venom. The elucidation of both N-terminal and internal stretches of amino acids will facilitate the future cloning and expression of *Oa*V proteins and this may confirm the suspected homologies outlined here and provide viable quantities of individual components for biological activity screening.

CHAPTER 4

THE C-TYPE NATRIURETIC PEPTIDE FROM *O. ANATINUS* VENOM: STRUCTURE, PHARMACOLOGY AND PATHOPHYSIOLOGICAL PROPERTIES

Introduction

When administered systemically to experimental animals, *O. anatinus* venom (*OaV*) produces hypotension, peripheral vasodilatation (Kellaway and LeMessurier, 1935) and gives rise to a sustained, tonic relaxation of the rat uterus *in vitro* (de Plater, Hons Thesis, ANU 1993). The latter activity is associated with a 4.2 kDa peptide which has been demonstrated to be a C-type natriuretic peptide (ovCNP-39) (de Plater *et al.*, 1995). This chapter describes the purification and structural characterisation of ovCNP-39 and addresses the possibility that it may contribute to the pathophysiological effects of *O. anatinus* venom (*Oa*V).

In mammals, three classes of natriuretic peptides have been described: atrial natriuretic peptide (ANP), which is predominantly produced by the cardiac atria (de Bold *et al.*, 1981); brain natriuretic peptide (BNP), originally isolated from porcine brain (Sudoh *et al.*, 1988) but predominantly produced by the cardiac ventricles; and C-type natriuretic peptide (CNP), the distribution of which includes the brain and endothelium (Sudoh *et al.*, 1990; Suga *et al.*, 1993). All forms incorporate a 17-residue intra-molecular disulphide loop, with ANP and BNP characteristically having both C- and N- terminal sequence extensions beyond the loop, while CNP extends only N-terminally. Two principal post-translational cleavage products of a common CNP precursor have been identified in eutherian mammals: CNP-22 (Sudoh *et al.*, 1990) and the less abundant N-terminally extended CNP-53 (Minamino *et al.*, 1990).

Although the normal physiological role of the natriuretic peptides, in particular CNP, is unclear, their natriuretic, diuretic and vasorelaxant properties suggest that they play a role in the control of blood pressure and fluid/salt homeostasis (Nakao *et al.*, 1992). Natriuretic peptides produce their vasorelaxant/hypotensive effects by elevating intracellular cyclic guanosine 3',5'- monophosphate (cGMP) in vascular smooth muscle cells. This occurs *via* the activation of two classes of receptor, ANP_A and ANP_B, both of which function as particulate guanylate cyclases (Chang *et al.*, 1993, Koller *et al.*, 1991). CNP acts *via* ANP_B (Koller *et al.*, 1991) whereas ANP and BNP act *via* ANP_A receptors (Schulz *et al.*, 1989; Lowe *et al.*, 1989). A third class of receptor, ANP_C, has no guanylate cyclase domain and is believed to be important for the metabolic clearance of ANP (Almeida *et al.*, 1989).

CNP has been shown to stimulate cGMP production in uterine tissue and ANP_B receptors, which are selective for CNP, have been identified in the rat uterus (Dos Reis *et al.*, 1995). To confirm that ovCNP-39 is truly responsible for the uterus relaxing effect of *Oa*V, the potency of both isoforms of *ex vivo* material were compared with that of synthetic ovCNP-39. In addition, the relative potencies of synthetic ovCNP-39, CNP-22 and ANP in relaxing the rat uterus have been investigated. This is of particular interest since ANP is regarded as a potent inhibitor of rat uterine contractions (Bek *et al.*, 1988; Potvin and Varma, 1990).

ovCNP-39 may, *via* guanylate cyclase activation in vascular smooth muscle, contribute to the hypotension and vasodilatation which accompanies *Oa*V administration. The ability of this peptide to elevate cGMP in vascular smooth muscle cells has therefore been investigated. The possibility that ovCNP-39 can cause plasma extravasation (oedema) and mast cell degranulation has also been addressed. Such an action would be consistent with the properties of ANP and CNP-22 which are known both to participate in fluid and electrolyte homeostasis (Flynn *et al.*, 1983; Morita *et al*,

1992) and to induce mast cell degranulation (Opgenorth, *et al.*, 1990; Yoshida *et al.*, 1996). The amino acid sequences of natriuretic peptides indicate that they are members of the polybasic, amphiphilic group of peptides which include mastoparan, substance P and kinins (Aridor *et al.*, 1993). These peptides degranulate mast cells in a receptor-independent fashion by interacting directly with the heterotrimeric G protein, G_{i3} (Aridor *et al.*, 1993). The possibility that ovCNP-39 induces histamine release from mast cells in a similar, receptor-independent manner has been investigated using ovCNP-39 peptide fragments.

Methods

Peptide purification

Whole *Oa*V was diluted in 0.1 M ammonium acetate, pH 5.0 and subjected to ultrafiltration using a 10 kDa nominal molecular weight filter (Centricon 10, Amicon). The filtrate was recovered and fractionated by reversed phase HPLC (RP-HPLC) using a C2/C18 column (μRPC SC2.1/10, Pharmacia). Eluent A consisted of 0.1 % trifluoracetic acid (TFA) in H_2O and eluent B consisted of 0.1 % TFA in CH_3CN. Peptides were eluted using a linear gradient of 0-60 % B over 20 min at a flow rate of 250 μl min^{-1}.

Capillary electrophoresis

O. anatinus venom and synthetic peptides were analysed by capillary electrophoresis, using a Beckman P/ACE™ 2000 system. Peaks were monitored by UV detector at a wavelength of 214 nm. 20 μl of a 1:10 dilution of whole venom was used and synthetic ovCNP-39 was employed as an external retention-time standard. Separation was achieved over 20 min at a field strength of 500 V cm^{-1} using 20 mM sodium citrate/20 mM MES (2-[*N*-morpholino]ethanesulphonic acid), pH 6.0, and a 27 cm eCAP™ neutral capillary

(Beckman). Peak areas were obtained by integration and a ratio of a given peak area to the total area above baseline was used to determine the relative concentration of ovCNP-39.

Isolated rat uterus preparation

The effect of *Oa*V fractions on the isolated rat uterus was examined. This preparation was initially used to screen the venom for bradykinin-like activity but as described above it was found that *Oa*V relaxed, rather than contracted, the rat uterus. The preparation was quite sensitive to *Oa*V and therefore its use as a bioassay was retained. A method based on Li and Rand (1989) and Martinez-Mir *et al.* (1990) was used. Diethylstilboestrol (DES, 5 mg kg^{-1}) was administered intraperitoneally to female Wistar rats (300-500 g) 24 hours before each was anaesthetised with 4% halothane in O_2, killed by decapitation and exsanguinated. Spontaneous contractions were often observed in uteri from rats not treated with DES, making it difficult to measure changes in baseline tension and DES treatment was therefore used routinely. Uteri from DES-treated rats had much less spontaneous activity at room temperature and this may be related to the fact that oestrogen has an inhibitory effect on prostaglandin-induced contractions in this preparation (Perusquia and Kubli-Garfia, 1992). An abdominal incision was made and the intestine pulled to one side exposing the uterine horns which were trimmed of both mesentery and fat and divided into segments 1 cm in length. Each segment was suspended in an organ bath by tying a loop at each end with a surgical thread and looping one end over a rigid wire at the bottom of the bath and tying the other end to an isotonic strain gauge linked *via* a MacLab™ digital/analog interface to a MacIntosh computer. A resting tension of 1 g was applied and 30 min allowed for equilibration. The bath solution contained (mM) 164.0 NaCl, 5.6 KCl, 0.6 $CaCl_2$, 2.7 glucose and 6 $NaHCO_3$ and was bubbled with 95% O_2 / 5% CO_2. Stable

tonic contractions were obtained by adding 50 mM KCl and tension was monitored continuously. Relaxation was expressed as a percentage of the KCl-induced contraction. All experiments were conducted at room temperature (20 - 23 °C).

Synthesis of peptides

CNP-22 (human, porcine and rat forms are identical), ovCNP-39, ovCNP-39(1-17) and ovCNP-39(18-39) were synthesised using an Applied Biosystems 470A solid phase peptide synthesizer using FMOC/NMP chemistry. Disulphide bridges were formed by potassium ferricyanide oxidation[12]. All peptides were purified by preparative RP-HPLC (BioRad Model 700 Gradient Module) using a Dynamax C8 column (300A, 21.4 mm x 25 cm, Ranin Associates) and analyzed by amino acid analysis and TOF-MALDI mass spectrometry to confirm authenticity. Rat α-ANP(1-28) was purchased from Auspep (Melbourne, Australia). The sequences of the peptides used are given in Table 4.1.

Table 4.1. Amino acid sequences of natriuretic peptides and fragments

ovCNP-39	LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGS**T**SGLGC
CNP-22*	GLSKGCFGLKLDRIGS**M**SGLGC
ovCNP-39(1-17)	LLHDHPNPRKYKPANKK
ovCNP-39(18-39)	GLSKGCFGLKLDRIGSTSGLGC
rat α-ANP(1-28)	SLRRSSCFGGRIDRIGAQSGLGCNSFRY

Amino acid difference between ovCNP-39 and CNP-22 is indicated in boldface
*identical for human, rat and pig

Preparation of rat aortic smooth muscle cell cultures

Smooth muscle cells were prepared using a method based on that described by Mar-

[12] Ferricyanide oxidation was carried according to the protocol described in the Applied Biosystems Inc. manual, *Strtegies in Peptide Synthesis - Introduction to cleavage.* pp. 58-59

sault *et al.* (1991) and Schweitz *et al.* (1992). Segments of descending thoracic aortae were excised from male Wistar rats (6-10 weeks old) and placed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.1 % collagenase (Type I, Sigma) for 2 h at 37 °C. Connective tissue was carefully removed under a dissecting microscope and each aortic segment was opened longitudinally and the intimal surface removed by gentle scraping with a pasteur pipette. The denuded pieces of tissue were then transferred to a sterile centrifuge tube containing Ca^{2+}- and Mg^{2+}-free PBS and 0.02% elastase (Type IV from Porcine pancreas, Sigma) and incubated at 37 °C for 30 min. Myocytes were mechanically dissociated by trituration using a pasteur pipette. Intact pieces of tissue were transferred to a fresh solution of elastase, re-incubated at 37 °C for a further 30 min and triturated again. The resulting supernatants were pooled and the dissociated cells recovered by centrifugation at 125 ***g*** (1000 rpm) for 5 min and resuspended in Medium 199 (GIBCO), supplemented with 20 % fetal bovine serum (GIBCO), 200 units ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin. The cell suspension was evenly distributed into the collagen-coated wells of a 24-well tissue culture plate. Plates were incubated at 37 °C in 95% O_2 / 5% CO_2. Cells were passaged every 5 days by washing confluent layers once with Ca^{2+} and Mg^{2+} free PBS and then incubating the cells with the same solution but containing 0.1 % trypsin, at 37 °C for 10 min. The resulting suspension was distributed into new collagen-treated tissue culture plates as described above. Experiments were conducted using cells from passage number 5 as these have previously been shown to predominantly express ANP_B receptors (Suga *et al.*, 1992).

Although removal of the intima should result in a pure smooth muscle cell culture (Chamley-Campbell *et al.*, 1979), it was not possible to determine whether this was accomplished without resorting to electron microscopy. Under phase-contrast microscopy, the morphology of individual vascular smooth muscle cells is difficult to

discern in confluent cultures and, even if this could be accomplished, they are morphologically similar to fibroblasts (Ross, 1971). Therefore, to confirm that the cultures contained predominantly smooth muscle cells, an FITC-conjugated antibody against smooth muscle α-actin, was used. This antigen is specifically found in smooth muscle cells (Skalli *et al.*, 1986). Confluent cell layers were dissociated according to the passaging method described above and the resulting cell pellet resuspended in PBS and placed on ice. Methanol (cooled in liquid N_2) was added drop-wise to a final concentration of 90 % (v/v) as the tube was vortexed gently. The cells were incubated for 30 min at -20 °C and collected by centrifugation, the supernatant was removed and the cells rinsed once with PBS and once with PBS containing 0.1% sodium azide and 0.1% BSA (PBA), suspended in 0.1% Triton/PBA, incubated on ice for 3 min and washed with PBA. The cells were resuspended in PBA containing 0.5 % (v/v) anti-α-smooth muscle actin (FITC conjugate, Molecular Probes, Inc.) and incubated for 1 h at 4 °C. Fluorescence-activated cell sorting (FACS) analysis revealed that 87 % of cells were immunoreactive to anti-smooth muscle α-actin, thereby confirming that the culture contained predominantly smooth muscle cells.

Cells were counted in confluent layers by fluorescent staining of nucleic acids using the CyQuant™ Cell Proliferation Assay Kit (Molecular Probes, Inc.). A standard curve was generated by dissociating cells with trypsin followed by haemocytometer counting. Cells, at defined concentrations, were incubated with the CyQuant™-GR/Lysis buffer following freeze-thawing and were vortexed. Cells were aliquoted into the wells of a 24-well tissue culture plate and the fluorescence of each well measured using a fluorescence microtitre plate reader with excitation and emission wavelengths set at 480 nm and 520 nm, respectively. A standard curve of fluorescence *versus* number of cells was constructed. For determining cell concentrations in confluent layers, media was removed from the wells by gently inverting each plate.

Plates were then frozen at -70 °C, thawed and the CyQUANT-GR/Lysis buffer added to each well. Fluorescence was read after 5 min, as described above and the number of cells determined from the standard curve.

cGMP measurements

Incubations were performed according to Schweitz *et al.* (1992). Cells were pre-incubated in 1 ml Earle's salt solution (mM: 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 5 glucose, 50 HEPES, pH 7.4), containing 0.3 mM of the phosphodiesterase inhibitor, isobutylmethylxanthine (Sigma), for 10 min at 37 °C. Peptides were added and the plate was incubated for a further 10 min at 37 °C. The incubation solution was then replaced with 200 µl of a 2:1 ethanol/5 mM EDTA solution and the plate incubated at 4 °C for 2 h. The contents of each well was transferred to an Eppendorf tube and dried *in vacuo*. The extracts were then resuspended in 200 µl 0.05 M Tris, 4 mM EDTA, pH 7.4, and cGMP concentrations determined by competitive radioimmunoassay using a cGMP [^{3}H] assay kit (Amersham). [^{3}H]cGMP was added to each assay tube, followed by 0.05 M Tris, pH 7.5 and 4 mM EDTA. 0.5-8 pmol cGMP standards (to construct a standard curve) and vascular smooth cell extracts were then added to the tubes, followed by antiserum. The tubes were incubated in the refrigerator for 1.5 h to allow the antibody to bind. The antibody-cGMP complex was then precipitated using a 60 % saturated $(NH_4)_2SO_4$ solution and incubating the tubes in an ice-bath for 5 min followed by centrifugation. The supernatant was carefully decanted and the pellet resuspended in MilliQ H_2O. The samples were mixed thoroughly with liquid scintillant (PCS, Amersham) and counted in a Packard Tri-Carb 460 scintillation counter. C_0, cpm in the absence of unlabelled cGMP, was determined by subtracting the blank counts per minute (cpm) from the cpm tubes containing only the antibody and [^{3}H] cGMP. C_x, or cpm bound in the presence of standard or cGMP from smooth muscle

cell extracts, was determined from the cpm for tubes containing these. C_o/C_x was calculated and plotted against pmol cGMP standard to generate the standard curve. The pmol cGMP from the vascular smooth muscle cell extracts was determined using the corresponding C_o/C_x values and this standard curve.

Isolation and purification of peritoneal mast cells

Mast cells were obtained from male Wistar rats (300-500 g). Each animal was anaesthetised with halothane, decapitated and an incision made in the abdominal cavity. The peritoneum was washed using 8 ml phosphate buffered saline containing 10 U ml^{-1} heparin. Cells were collected by centrifugation at 4 °C for 10 min at 500 ***g*** (2500 rpm) and the resulting crude cell pellet (containing 5-10 % mast cells) was resuspended in 3 ml HEPES buffer containing (in mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 0.3; $MgCl_2$, 1.0; NaH_2PO_4, 0.4; glucose, 5.6; HEPES, 10, pH 7.4 and 0.1% bovine serum albumin, and layered over 2ml 30% Ficoll (in HEPES buffer/BSA). Following centrifugation at 4 °C for 10 min at 180 ***g*** (900 rpm) the Ficoll layer was diluted in 8 ml HEPES buffer (without BSA) and the mast cells collected by centrifugation at 500 ***g*** (2500 rpm) and resuspended in HEPES buffer (without BSA) at a concentration of 10^5 cells ml^{-1}. Mast cell purity was greater than 80% as determined by cell morphology and haemocytometer counting. Mast cells from several rats were pooled for each day's experiments.

Assay of mast cell histamine release

Mast cells were aliquoted into tubes containing peptides in 50 µl HEPES buffer (without BSA) to give a final cell concentration of $5x10^4$ per ml. The tubes were vortexed briefly and incubated at 37 °C for 15 min after which they were placed on ice and centrifuged at 4 °C for 10 min at 500 ***g*** (2500 rpm). The supernatant was removed

and placed in sample tubes. Histamine released into the supernatant was assayed by HPLC (LC1090, Hewlett Packard) using pre-column derivitisation with *ortho*-pthalaldehyde (OPA with 3-mercaptopropionic acid in 0.4 M sodium borate, pH 10.4, Hewlett-Packard): 1 µl supernatant was mixed with 7 µl sodium borate (pH 10.4, Hewlett Packard) and 3 µl OPA reagent using the auto-sampling facility of the AminoQuant Sysem. This was injected onto an ODS-Hypersil C18 (5 µm, 20 x 2.1 mm, Hewlett-Packard) guard column, which provided good separation in a short time with sensitivity in the pmol μl^{-1} range. Buffer A consisted of 0.1 M sodium acetate, 1.3 mM triethylamine (Pierce, HPLC grade) and 0.4 % (v/v) tetrahydrofuran, titrated to pH 7.2 with acetic acid and Buffer B consisted of acetonitrile:methanol:buffer A (10:10:5). Three histamine-OPA adducts (Ronnberg *et al.*, 1984) were eluted using a 5 min linear gradient of 0-100% B at a flow rate of 450 µl min^{-1} and detected using an HP 1046 (Hewlett-Packard) fluorescence detector with excitation and emission wavelengths of 330 nm and 450 nm, respectively. The adduct giving rise to the largest peak was used for quantitation as this was unaffected by peptide and other material in the supernatant.

Measurement of oedema

The ability of peptides to produce oedema was determined using the rat hind paw. Female Wistar rats (300-500 g) were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p., with supplementary doses to maintain anaesthesia). Synthetic peptides, dissolved in 50 µl phosphate buffered saline (PBS), were introduced into one hind paw by way of subplantar injection. The PBS vehicle was injected into the opposite paw as a control. Three animals were used for each peptide concentration (a total of 18 rats). Increases in paw volume occurring as a result of oedema were measured plethysmographically using the method of Van Arman *et al.* (1965). An apparatus was constructed consisting of a halved 50 ml syringe barrel, half-filled with mercury and

attached to a blood pressure transducer (Gould Statham P23ID) *via* a tube filled with H_2O. The blood pressure transducer was connected to a blood pressure monitor/amplifier (constructed by JCSMR workshop) which was attached to a MacIntosh computer *via* a MacLab™ digital/analog interface and monitored using the Chart™ program. The entire apparatus was mounted on a perspex stand and experiments were carried out under a fume-hood (to avoid the risk of mercury vapour inhalation). A line was marked above the topmost callus pad on each rat hind-paw and the paw was immersed to that line. The mercury displaced by the paw immersion caused a pressure increase which was transmitted to the blood pressure transducer by the H_2O impinging upon the transducer diaphragm. Thus increases in paw volume resulting from oedema produced an increase in the recorded mercury displacement and this was shown in preliminary experiments to be reproducible. Oedema was monitored for three hours, after which time the rats were sacrificed by pentobarbitone overdose.

Statistics

Values are expressed as means ± SEM. Dose-response data was fitted by the method of least squares using a four-parameter logistic function (Microcal Origin © program, Ver. 3.54, Microcal Software Inc.). Data were analysed using the Student's t-test for comparing two EC_{50} or paw volume means, or one way analysis of variance for comparing multiple EC_{50} values. Differences were considered significant if $p<0.05$.

Results

Purification of the CNP-like peptide

Material passing through a 10 kDa nominal molecular weight exclusion ultrafiltration membrane was subjected to RP-HPLC and the resulting fractions were assayed for uterus relaxant activity (Fig. 4.1). Maximum activity was associated with two A_{214}

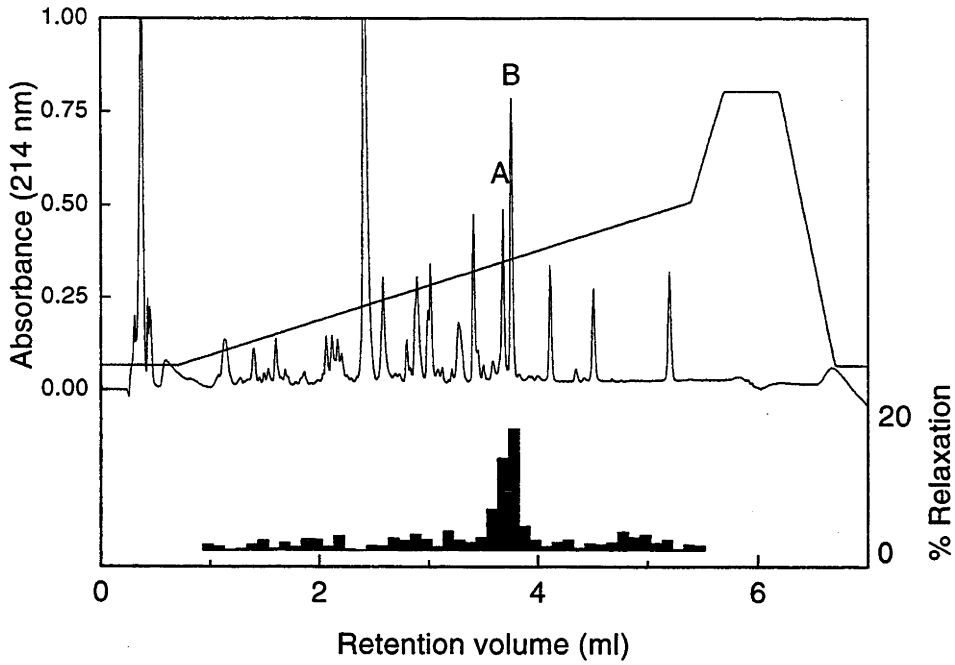


FIG. 4.1. Reversed phase HPLC chromatogram of *O. anatinus* venom 10 kDa ultrafiltrate showing rat uterus relaxing activity associated with each 100 µl fraction. Maximum activity was associated with the two peptides designated A and B.

peaks. MALDI-TOF mass spectrometric analysis of these peaks revealed $[M+H]^+$ masses of 4207.9 and 4208.3, respectively. Each peptide was homogeneous by SDS-PAGE, RP-HPLC and TOF-MALDI mass spectrometry.

Structure

Both active peptides were subjected to direct N-terminal sequence analysis. In addition, fragments generated by digesting the peptides with endoproteinase Arg-C (Fig. 4.2) and chymotrypsin (Fig 4.3) were sequenced. The sequences obtained for each peptide were identical, despite the fact that both the N-terminal Arg-C and chymotryptic fragments of the isoforms differed in retention times. The elucidated primary structure, shown in Fig. 4.4, consisted of 39 residues which gives a calculated $[M+H]^+$ mass of 4208.5. The homology between these peptides and members of the C-type natriuretic peptide (CNP) family (Fig. 4.5) led to the designation of ovCNP-39 (*Ornithorhynchus* venom C-type natriuretic peptide). The isoforms, named according to their order of RP-HPLC elution, are designated as ovCNP-39A and ovCNP-39B, respectively. The ovCNP-39 sequence is characterised by a non-conservative Met $\rightarrow$ Thr substitution at position 34 in comparison to the consensus eutherian form. This lies within the highly conserved 17 residue "loop" which is between the two Cys residues of natriuretic peptides.

In order to elucidate the basis for the heterogeneity between ovCNP-39A and ovCNP-39B, mixtures of the purified peptides and synthetic ovCNP-39 were analysed by RP-HPLC. As shown in Fig 4.6, ovCNP-39A co-eluted with the synthetic material whereas ovCNP-39B eluted as a separate peak. There was apparently no dynamic exchange between the isoforms: upon re-chromatography, each eluted as a single peak with a conserved retention time. This was also observed after incubating the peptides in 6 M guanidine-HCl at 100 °C for 1 h. Chymotryptic mapping of each (disulphide-

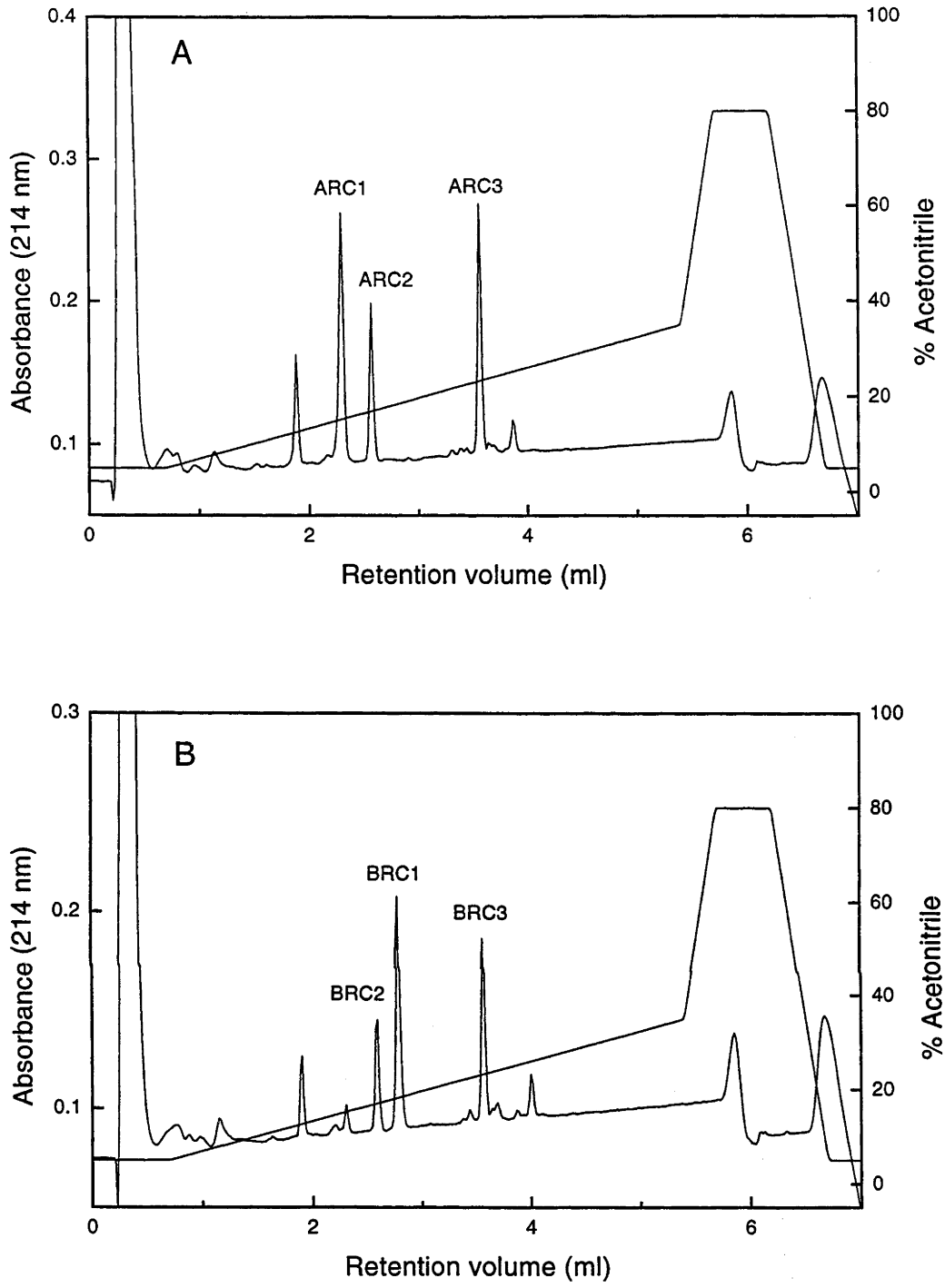


FIG. 4.2. Endoproteinase Arg-C (RC) digestion of active material, peak A (**A**) and peak B (**B**). The material was reduced, S-pyridylethylated and incubated with Endoproteinase Arg-C for 3 hours at 37°C followed by reversed phase HPLC analysis. Peak labels correspond to regions of elucidated sequence shown in Fig. 4.4.

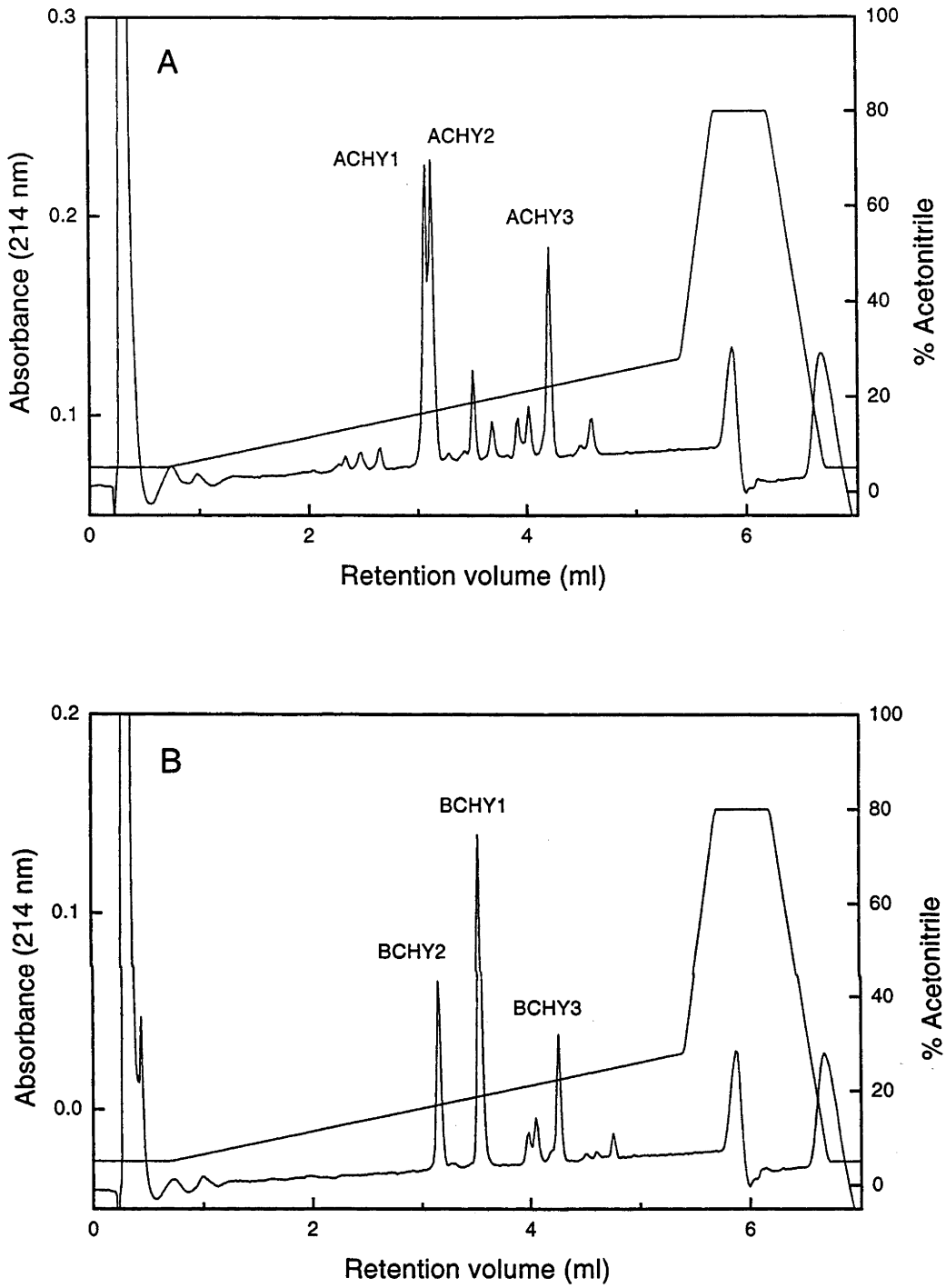


FIG. 4.3. Chymotryptic (CHY) digestion of active material, peak A (**A**) and peak B (**B**). The material was reduced, S-pyridylethylated and incubated with Endoproteinase Arg-C for 3 hours at room temperature followed by reversed phase HPLC analysis. Peak labels correspond to regions of elucidated sequence shown in Fig. 4.4.

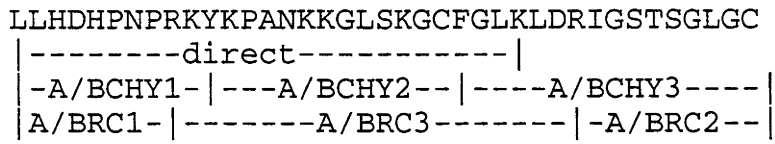


FIG.4.4. The elucidated amino acid sequence of the A and B material. The full, 39 - residue sequence was determined from the N-terminus of intact peptides and from chymotryptic (CHY) and Arg-C (RC) fragments. The sequences obtained for both peptides (A/B) were identical and the level of homology with C-type natriuretic peptides led to the designation of ovCNP-39 (*Orntihorhynchus* venom C-type natriuretic peptide, 39 residues).

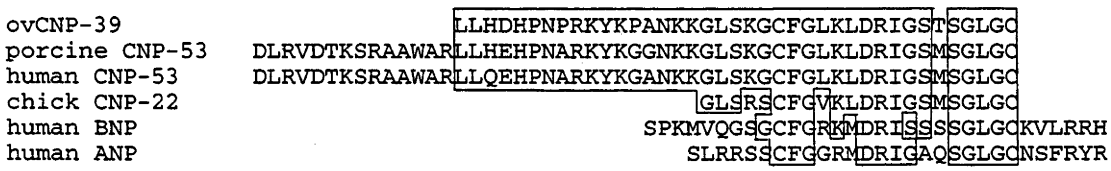


FIG. 4.5. Comparison of natriuretic peptide sequences. The C-terminal 22 residue portions of human and porcine CNP-53 are identical and correspond to CNP-22. Within this region, ovCNP-39 is characterised by a Met → Thr substitution at position 34. ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide.

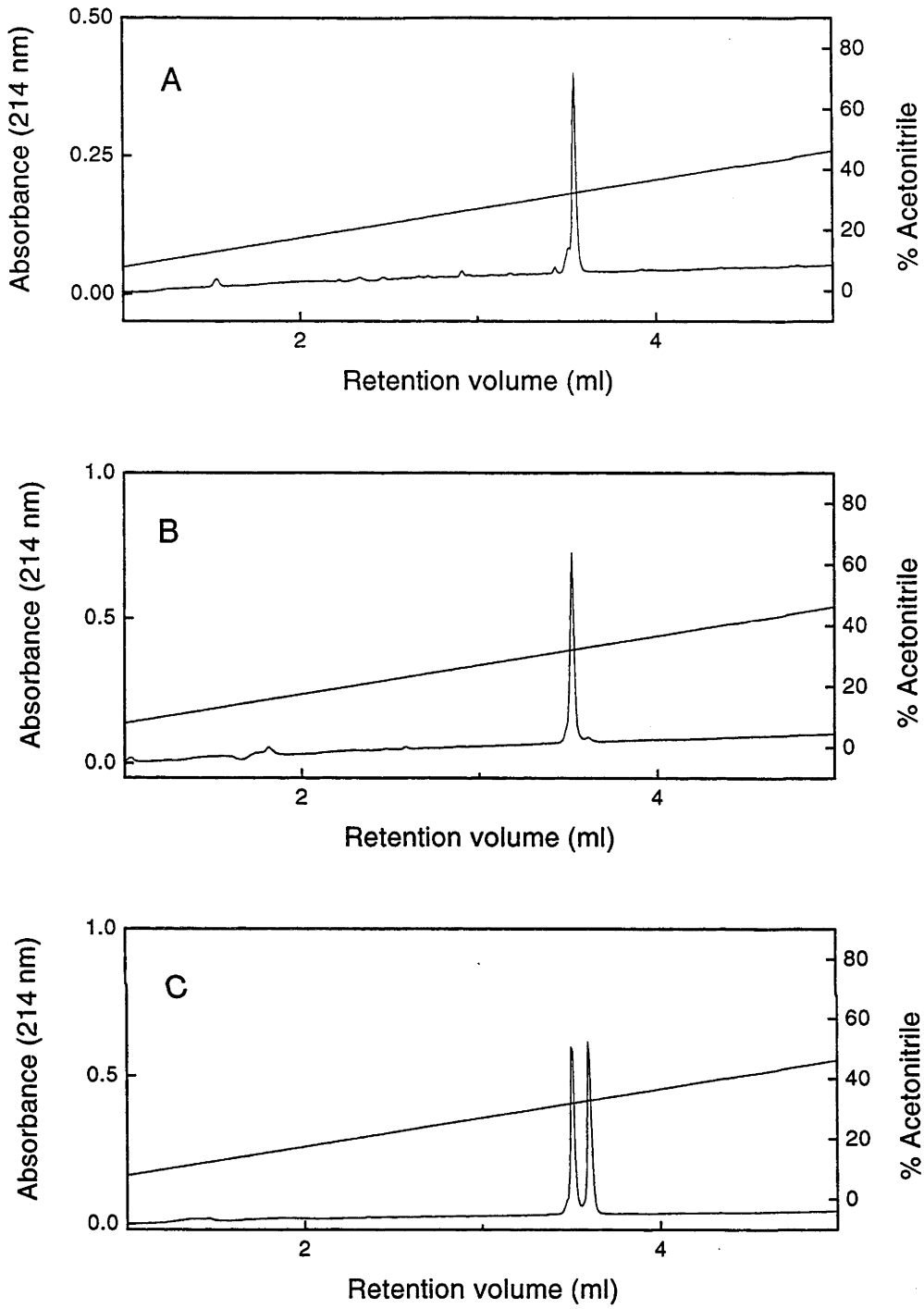


FIG. 4.6. HPLC co-elution analysis of synthetic ovCNP-39 (**A**), ovCNP-39A (**B**) and ovCNP-39B (**C**). Each of these peptides was mixed and injected simultaneously with synthetic ovCNP-39. ovCNP-39A (**B**) co-eluted with the synthetic material whereas ovCNP-39B (**C**) eluted differently. Synthetic ovCNP-39 is therefore similar to ovCNP-39A and the heterogeneity appears to be associated with the B isoform.

intact) isoform showed that the heterogeneity is associated with the N-terminal 11 residues (Fig. 4.7) because the N-terminal chymotryptic fragment of synthetic ovCNP-39 and ovCNP-39A eluted with comparable retention times, whereas the ovCNP-39B N-terminal chymotryptic fragment eluted somewhat later. By FAB mass spectrometry each of these N-terminal fragments had a mass of 1389.7 which is identical to the calculated mass. Further cleavage at Asp^4 with endoproteinase Asp-N proceeded in the N-terminal chymotryptic fragment derived from ovCNP-39A but not in that derived from ovCNP-39B (Fig. 4.8).

The proportion (w/w) of ovCNP-39 in whole venom was estimated at 0.086 using relative A_{214} peak area derived from capillary electrophoresis. As the detection was performed at 214 nm, the peak area resulting from each component of the venom is proportional to both its molecular mass and concentration, *i.e.* proportional to the total weight of each component. As most components of the venom are of a higher molecular weight than ovCNP-39 (see Chapter 3), the mass ratio of 0.086 thus obtained would be lower than the molar ratio. Therefore, while the relative mass fraction of ovCNP-39 represents 8.6% of venom components, it is likely to represent a substantially greater mole fraction.

Rat uterus activity

3 nM purified ovCNP-39A and ovCNP-39B produced a 37 ± 3 % (n = 3) and 41 ± 4 % (n = 3) relaxation of the KCl-contracted rat uterus, respectively (Fig. 4.9). This was comparable to the effect of 3 nM synthetic ovCNP-39, which produced a 43 ± 2 % (n = 4) relaxation, but differed significantly ($p < 0.01$) from that of the primary circulating form of rat ANP, α-ANP(1-28), of which 3 nM produced only a 13 ± 1 % (n = 4) relaxation (Fig. 4.9).

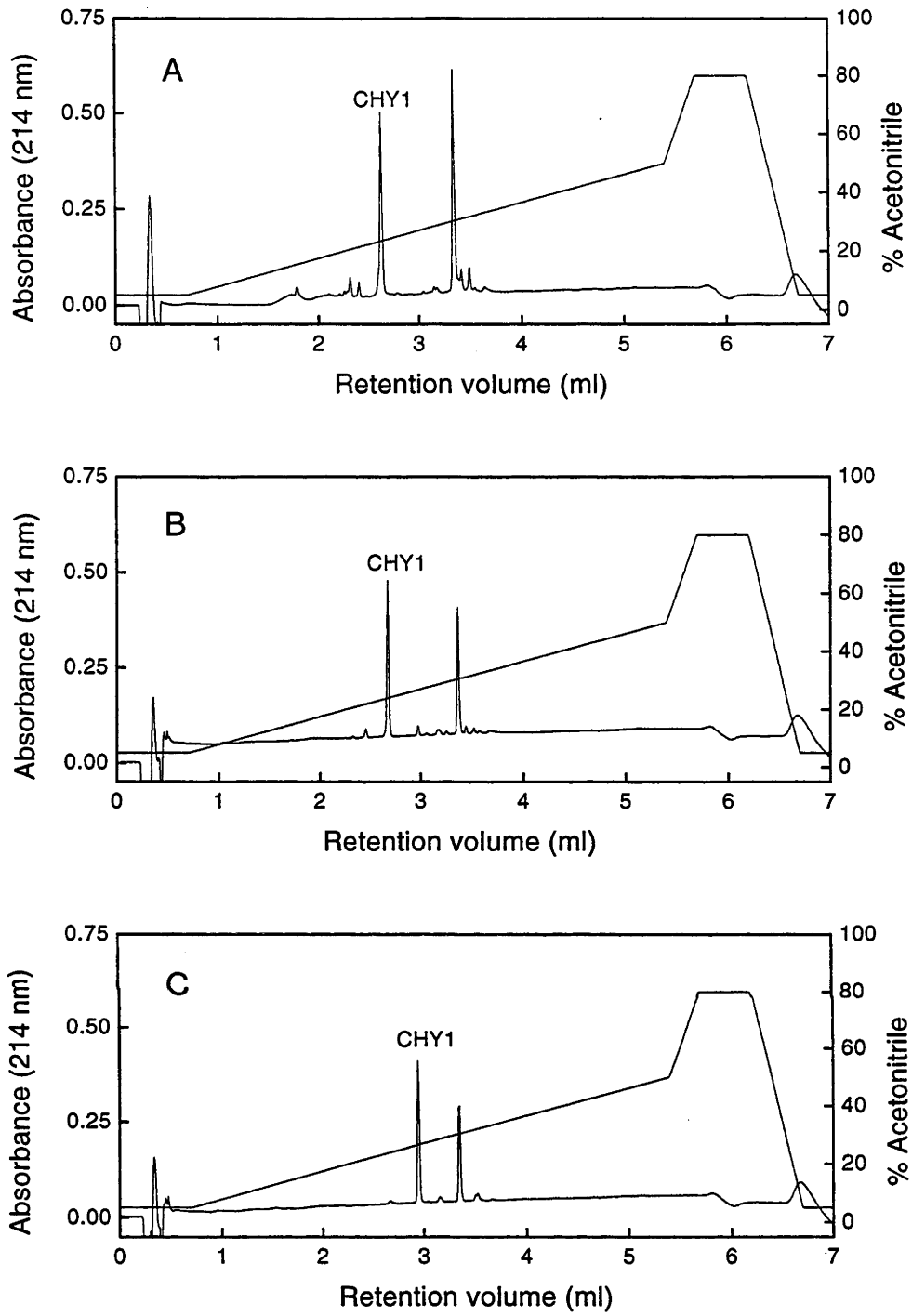


FIG. 4.7. HPLC analysis of chymotryptic fragments from synthetic ovCNP-39 (**A**), ovCNP-39A (**B**) and ovCNP-39B (**C**). Disulphide-intact material was digested with chymotrypsin. This resulted in two fragments: the N-terminus, which corresponds to CHY1 (see Fig. 4.4) and the C-terminal fragment which is made up of the CHY2 and CHY3 fragments (see Fig. 4.4) attached *via* a disulphide linkage. CHY1 resulting from ovCNP-39B (**C**) eluted later than CHY1 from both the synthetic peptide (**A**) or ovCNP-39A (**B**).

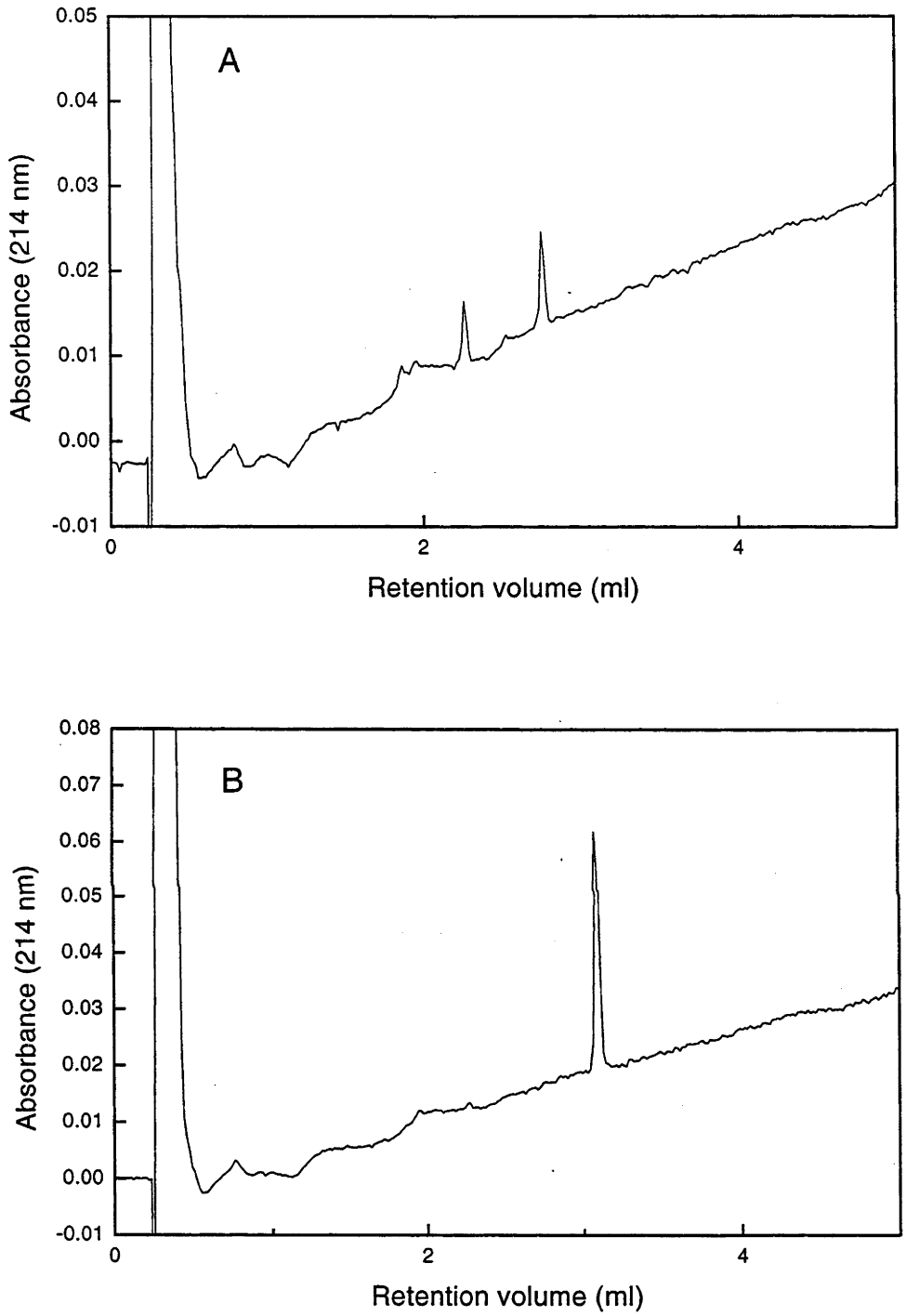


FIG. 4.8. Reversed phase HPLC analysis of peptides resulting from the Endoproteinase Asp-N cleavage of the N-terminal chymotryptic fragments derived from ovCNP-39A (**A**) and ovCNP-39B (**B**). Note that cleavage at Asp4 occurred in the ovCNP-39A fragment but not in the ovCNP-39B fragment.

Concentration-response curves for the relaxant effect of synthetic natriuretic peptides on the rat uterus are shown in Fig. 4.10. Synthetic ovCNP-39 relaxed the uterus more potently (EC_{50} = 4.8 ± 0.2 nM, n = 4) than ovCNP-39(18-39) (EC_{50} = 13.5 ± 2.1 nM, n = 4, $p < 0.05$), CNP-22 (EC_{50} = 14.1 ± 3.0 nM, n = 4, $p < 0.05$), and rat α-ANP1-28 (EC_{50} = 37.1 ± 2.4 nM, n = 4, $p < 0.01$). Full concentration-response data for the *ex vivo* ovCNP-39 isoforms on the rat uterus were not obtained due to material limitations.

Elevation of cGMP in cultured aortic smooth muscle cells

The effect of purified *O. anatinus* venom natriuretic peptides (ovCNP-39A and B) as well as synthetic ovCNP-39, ovCNP-39(18-39), CNP-22 and rat α-ANP(1-28) on cGMP formation in vascular smooth muscle cells (VSMCs) was assayed using cultured rat aortic myocytes. All of these peptides have an intact 17-residue disulphide loop which is the minimum requirement for natriuretic peptide receptor binding (Furuya *et al.*, 1992). The resulting concentration-response curves are presented in Fig. 4.11. After a 10 minute incubation with VSMCs at 37 °C, 1 μM ovCNP-39A induced a 55-fold (40.59 ± 6.32 pmol/10^6 cells, n=3) increase and 1 μM ovCNP-39B a 62-fold (45.94 ± 2.35 pmol/10^6 cells, n=3) increase in cGMP levels from resting levels (0.74 ± 0.07 pmol/10^6 cells, n=4). Similarly, 1 μM synthetic ovCNP-39, ovCNP-22 and CNP-22 produced 48, 54, and 53-fold increases in cGMP, whereas rat α-ANP(1-28) produced only a 4-fold (3.04 ± 0.10 pmol/ 10^6 cells, n=3) increase. The EC_{50} values for all the natriuretic peptides, other than ANP (for which no E_{max} was determined), were not significantly different ($p > 0.1$, n=3).

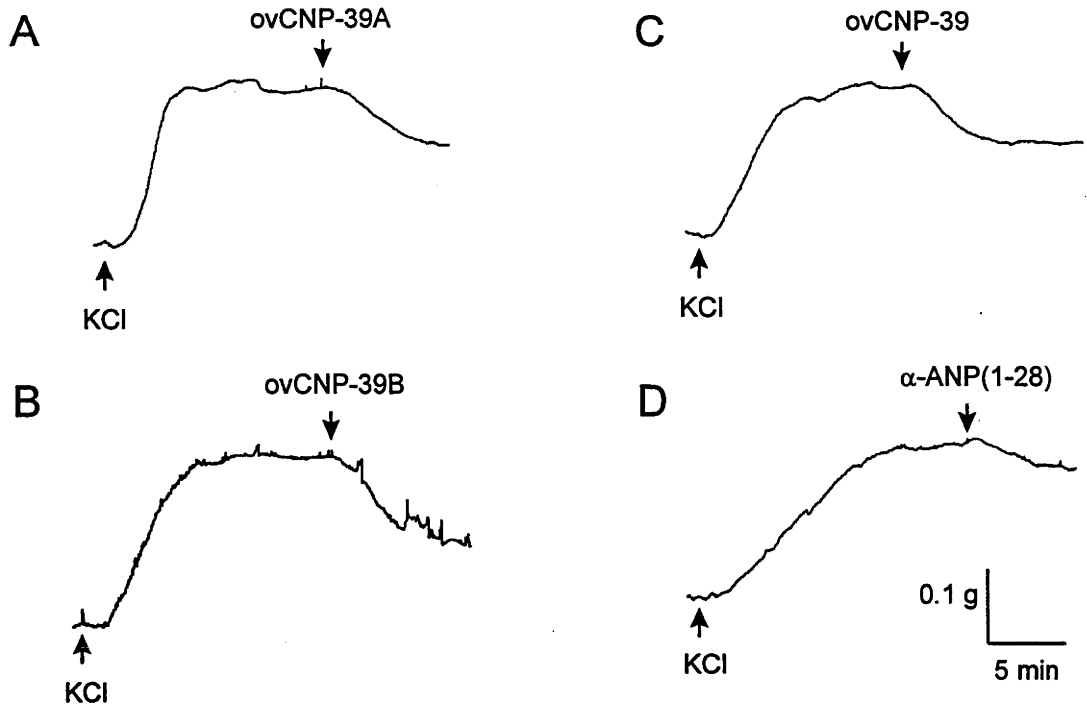


FIG. 4.9. Responses of the isolated rat uterus to 3 nM ex vivo ovCNP-39A (**A**), ovCNP-39B (**B**), 3 nM synthetic ovCNP39 (**C**) and 3 nM rat α-ANP(1-28) (**D**). The uterus was tonically pre-contracted with 50 mM KCl before the addition of each peptide.

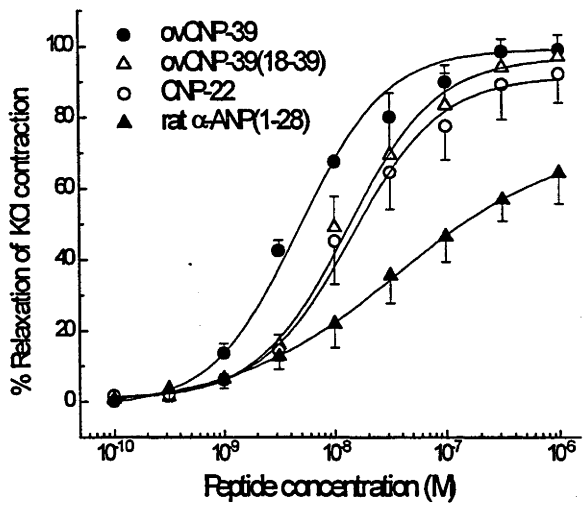


FIG. 4.10. Cumulative concentration-response curves for the relaxation of the KCl-contracted isolated rat uterus by natriuretic peptides. Peptides were added in increasing concentrations and isometric tension monitored continuously. Logistic functions were fitted to the data to obtain EC_{50} estimates.

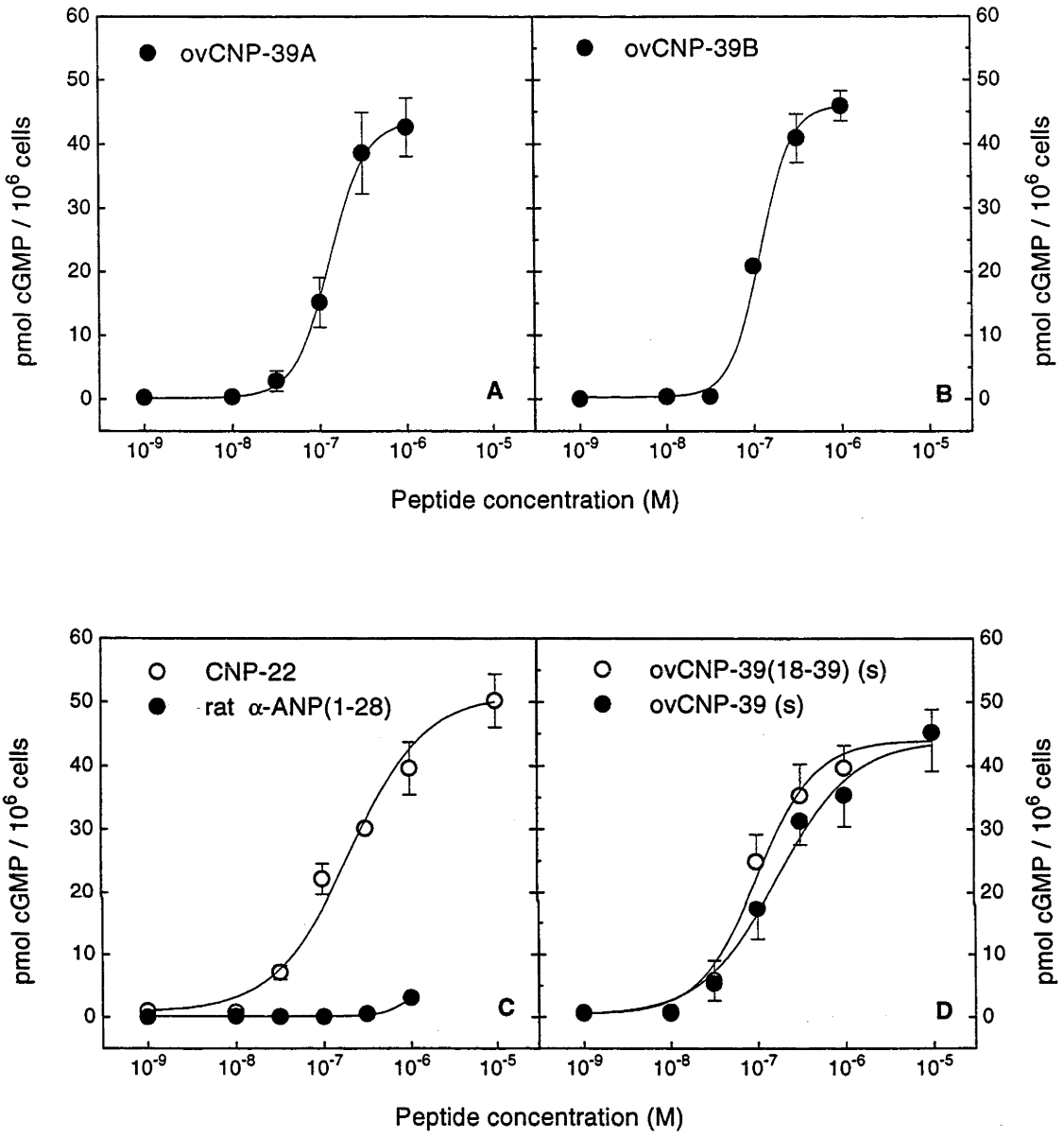


FIG. 4.11. The effect of ovCNP-39 and other natriuretic peptides on accumulation of cGMP in cultured vascular smooth muscle cells. Thoracic rat aortic smooth muscle cells from passage 5 were incubated with peptides for 10 min at 37 °C in Earle's salt solution with 0.3 mM isobutylmethylxanthine. ovCNP-39A (**A**) and ovCNP-39B (**B**) produced a concentration-dependent increase in cGMP which was similar to CNP-22 (**C**), synthetic ovCNP-39 (**D**) and the synthetic fragment, ovCNP-39(18-39) (**D**) but differed from that of ANP (**C**).

Oedemagenic effects

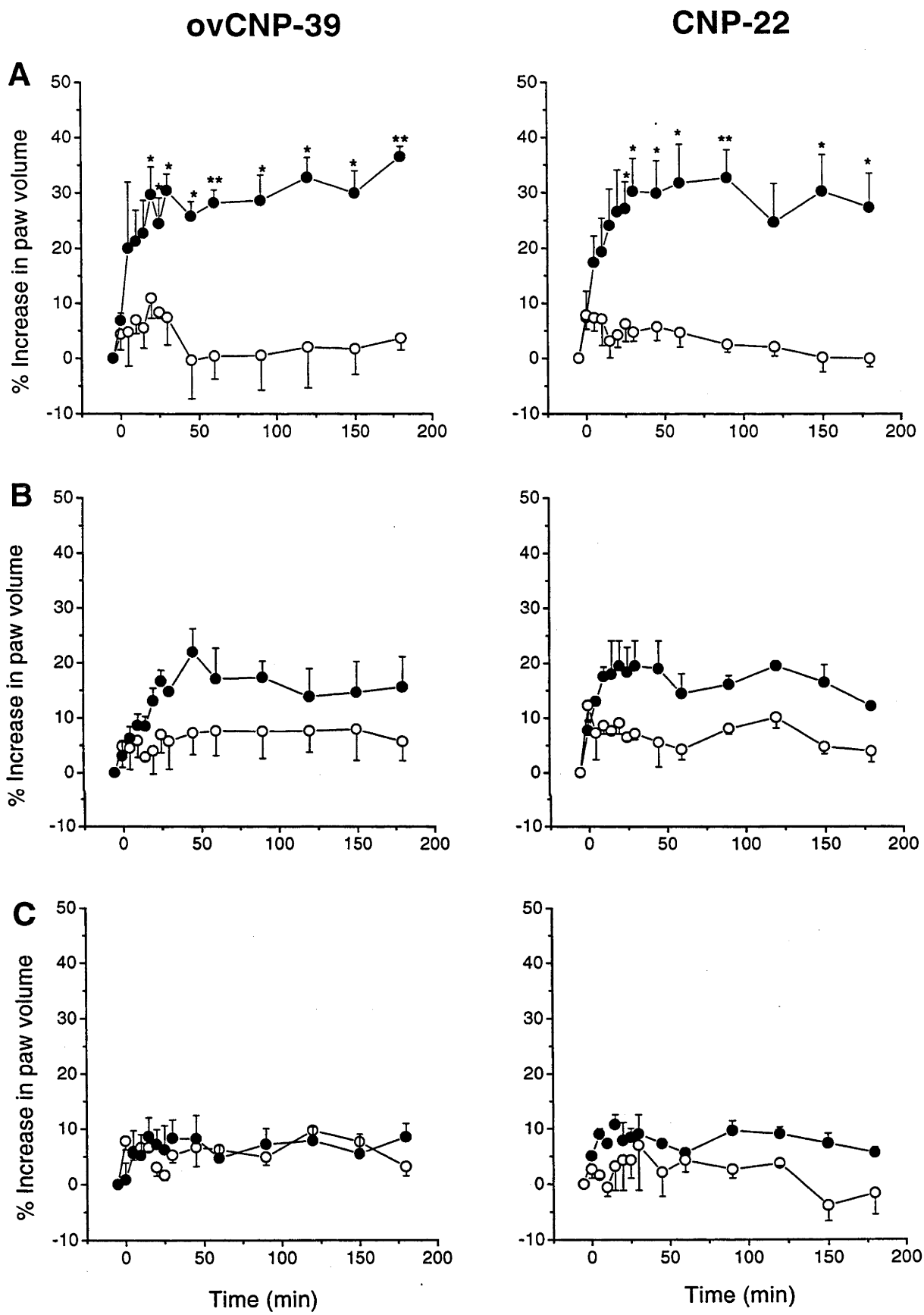
Synthetic ovCNP-39 and CNP-22 both produced oedema following subplantar injection into the rat hind paw (Fig. 4.12). 5 nmol (in a volume of 50 µl) of either ovCNP-39 or CNP-22, for each the highest concentration tested, led to a significant increase in paw volume after 20 min and this was sustained for up to 3 h. Maximal increases over contralateral, control paw volume were 37 ± 2 % (n = 3) for ovCNP-39 and 33 ± 5 % (n = 3) for CNP-22 (Fig. 4.12A). 250 pmol of each peptide (n = 3) produced a slight (though not significant) response (Fig. 4.12B), while 5 pmol (n = 3) produced a negligible response (Fig. 4.12C).

Mast cell histamine release

o-Pthalaldehyde (OPA) derivitisation of a histamine standard, followed by reversed phase HPLC, gave rise to three histamine-OPA adducts (Fig. 4.13A). The histamine-OPA fluorophore has been demonstrated previously to produce three peaks upon HPLC, which are believed to correspond to different stereoisomers which are readily inter-convertible (Ronnberg *et al.*, 1984). The adduct giving rise to the largest peak was used for quantitation as this was unaffected by peptide and other material in the supernatant.

Fig. 4.13B demonstrates the HPLC analysis of the total histamine released from mast cells by sonicating the cell pellet. An example of the HPLC analysis of baseline histamine release following a 10 min incubation at 37 °C in the absence of any secretogogue, is shown in Fig. 4.13C. An HPLC analysis of histamine release resulting from the incubation of mast cells with 0.1 mg ml^{-1} *Oa*V for 10 min at 37 °C is shown in Fig. 4.13D. It can be seen that this concentration of *Oa*V induces substantial release of histamine.

FIG 4.12. Oedemagenic effect of subplantar injections of ovCNP-39 and CNP-22 in the rat hind-paw. Peptides (in a 50 µl volume) were injected at concentrations of 10^{-4} M (A), 5 x 10^{-6} M (B) and 10^{-7} M (C) (filled circles) and the phosphate-buffered saline vehicle was injected into the contralateral paw as a control (open circles). Changes in paw volume were monitored plethysmographically over 180 min. * $p<0.05$, ** $p<0.005$.


ovCNP-39
CNP-22
A
B
C
% Increase in paw volume
Time (min)
50
40
30
20
10
0
-10
0
50
100
150
200
*
**

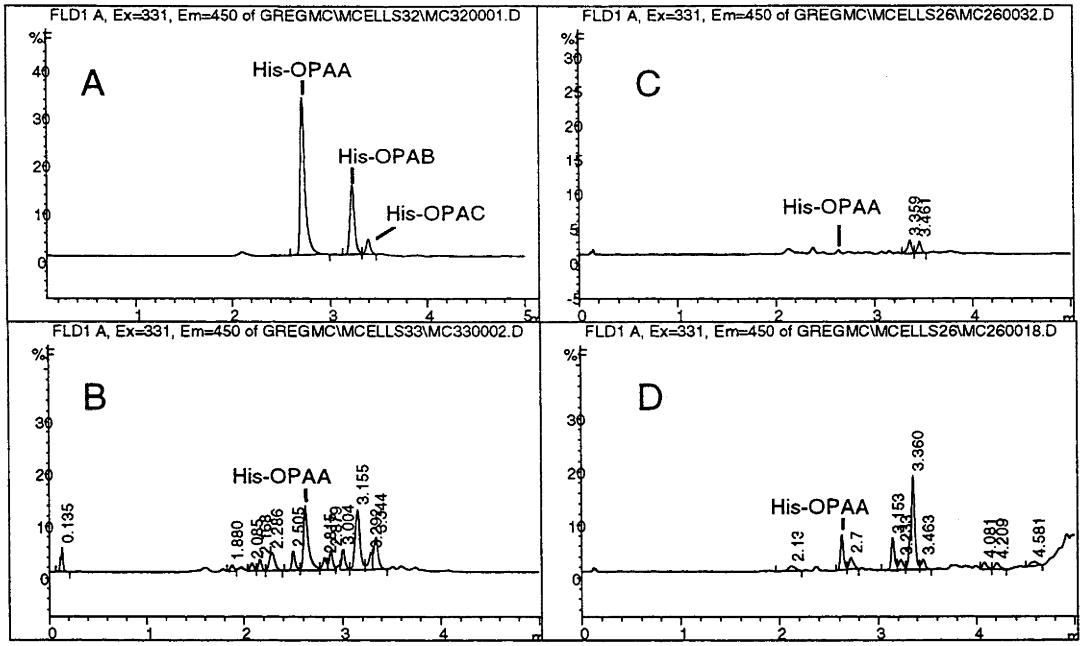


FIG. 4.13. Assay of histamine release from peritoneal mast cells. **A.** 100 pmol histamine standard giving rise to three histamine-OPA adducts, labelled His-OPA-A, B and C. Peak A was used for quantitation. **B.** Total mast cell histamine released by sonication. **C.** Basline histamine release over 10 min at 37 °C. **D.** Mast cell histamine released by incubation with 0.1 mg.ml^{-1} OaV for 10 min at 37 °C

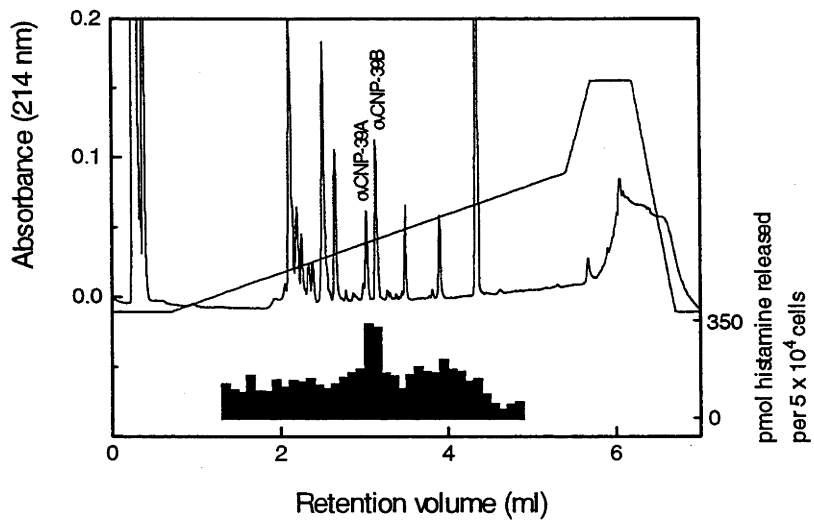


FIG. 4.14. Reversed phase HPLC chromatogram of a 10 kDa ultrafiltrate from *Oa*V. Each collected fraction (100 µl volume) was lyophilised and incubated with peritoneal mast cells for 10 min at 37 °C. The histamine release resulting from each fraction is shown in the histogram beneath the chromatogram.

A reversed phase chromatogram of a 10 kDa-cutoff ultrafiltrate of *Oa*V is shown in Fig. 4.14 and beneath it are the results of a preliminary screen of fractions for mast cell histamine-releasing activity. Fractions containing the ovCNP-39 isoforms, A and B, appeared to be most active. This led to the construction of dose-response curves for histamine release induced by synthetic[13] ovCNP-39, CNP-22 and α-ANP(1-28).

Synthetic ovCNP-39 produced a concentration-dependent release of histamine from purified peritoneal mast cells, with an estimated EC_{50} of 10.0 ± 3.8 μM (n = 4, Fig. 4.15). This was slightly (though not significantly) less potent than CNP-22 and rat α-ANP(1-28), which produced estimated EC_{50} values of 4.1 ± 0.5 μM (n = 4) and 2.9 ± 0.6 μM (n = 4), respectively (Fig. 4.15). However, α-ANP(1-28) showed significantly greater efficacy than either ovCNP-39 and CNP-22. 30 μM α-ANP(1-28) released 1333.9 ± 87.5 (n = 4) pmol histamine/$5x10^4$ cells, whereas 30 μM ovCNP-39 and CNP-22 released 889.0 ± 75.5 pmol ($p < 0.05$, n = 4) and 992.5 ± 109.5 pmol ($p < 0.05$, n = 4) / $5x10^4$ cells, respectively.

In order to investigate the possible existence of natriuretic peptide receptors on mast cells, the potency of ovCNP-39 and ovCNP-39(18-39) were compared with ovCNP-39(1-17), which lacks the disulphide loop essential for natriuretic peptide receptor binding. Concentration-response curves for the histamine-releasing activities of the ovCNP-39 fragments, ovCNP-39(1-17), ovCNP-39(18-39), and ovCNP-39 itself are presented in Fig. 4.16 (see Table 4.1 for sequences). There was no significant difference in the potency of these peptides (n = 4). ONP-39(1-17) and ONP-39(18-39) had estimated EC_{50} values of 6.6 ± 0.8 μM and 10.0 ± 1.8 μM, respectively.

[13] The relatively high concentrations of peptide required to elicit histamine release precluded use of the *ex vivo* peptide isoforms for these experiments.

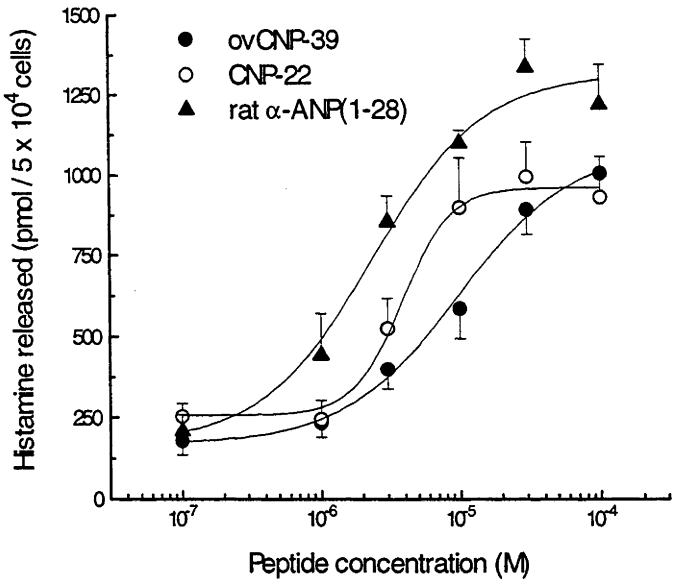


FIG. 4.15. Mast cell histamine release dose-response curves for natriuretic peptides. The ability of ovCNP-39 from OaV and endogenous CNP-22 and rat α-ANP(1-28) to release histamine from purified rat peritoneal mast cells was assessed over a range of concentrations.

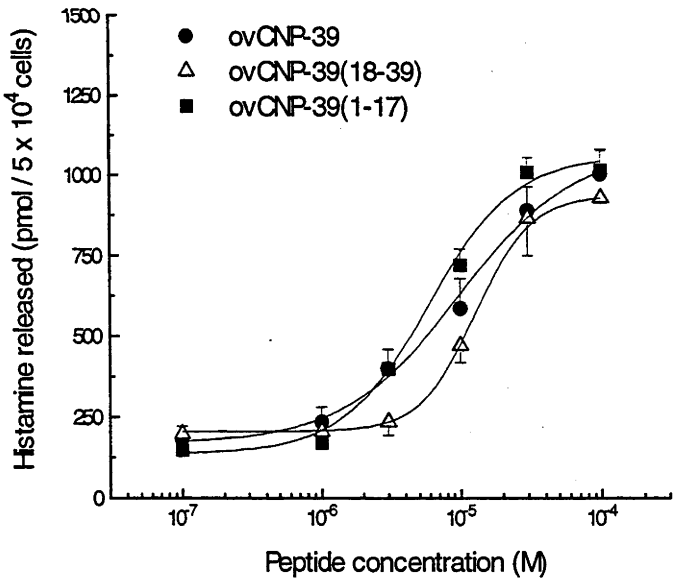


FIG. 4.16. Mast cell histamine release dose-response curves for ovCNP-39 fragments. The potency of intact ovCNP-39 and ovCNP-39 C-terminal (ovCNP-39(18-39)) and N-terminal (ovCNP-39(1-17)) fragments in releasing histamine from purified rat peritoneal mast cells were compared

Discussion

Structure

This chapter describes the characterisation of a 39-residue C-type natriuretic peptide, ovCNP-39, from *O. anatinus* venom which relaxes the rat uterus *in vitro.* Based on amino acid sequence homology, ovCNP-39 appears to be a typical member of the CNP class, although it is distinctive in that it comprises 39 residues and, *ex vivo,* exists as two isoforms. The identification of a C-type natriuretic peptide in *Oa*V is particularly interesting in that it constitutes a relatively high mole fraction of venom components.

The structure of CNP-22 is highly conserved between mammals to the extent that the human, porcine and rat forms are identical (Kojima *et al.*, 1991; Tawaragi *et al.*, 1990; Tawaragi *et al.*, 1991). CNP-53 shows slightly more heterogeneity (Tawaragi *et al.*, 1991) in its N-terminal sequence but as it is the loop structure which participates in ANP_B receptor activation, amino acid substitutions or extensions at the N-terminus do not affect activity (Furuya *et al.*, 1992). The high degree of similarity between ovCNP-39 and endogenous CNP-22 (the Met → Thr substitution, at position 17 of CNP-22, is the only difference) indicates the peptide is structurally a member of the C-type natriuretic peptide family.

The length of ovCNP-39 suggests that processing of the pro-peptide differs from that of CNP-22 and CNP-53. CNPs of varying lengths have also been isolated from the heart of several species of elasmobranch fish (Suzuki *et al.*, 1992; Takano *et al.*, 1994). These include CNP-38, CNP-39 (sequence is somewhat different from ovCNP-39, Suzuki *et al.*, 1992), CNP-21 and CNP-41 (Takano *et al.*, 1994). Retention of the typical proteolytic processing signal Lys-Lys, which precedes the C-terminal 22 residues in both ovCNP-39 and the elasmobranch cardiac forms suggests that the

proteolytic enzyme(s) which targets this site is either absent or inactive in both the platypus crural gland and the elasmobranch heart.

The difference between the two ovCNP-39 isoforms, which exhibited identical primary structures but eluted differently on RP-HPLC, was not resolved. The masses were identical and no dynamic exchange between the two forms was apparent, even under strongly denaturing conditions. This suggests an unusual, stable conformational difference between the isoforms, which has not been previously reported in natriuretic peptides. The heterogeneity was localised to the N-terminal 11 residues and apparently makes the His^3-Asp^4 bond refractory to cleavage by endoproteinase Asp-N in the B isoform. This stretch contains two prolines and it is therefore possible that the isoforms represent different proline *cis-trans* conformers, as has been reported in a number of peptides (Gesquiere *et al.*, 1989, O'Neal *et al.*, 1996). Such peptides have slow rates of isomerisation and can therefore be resolved on RP-HPLC as two peaks, one representing the *cis* conformer and the other the *trans* conformer (Gesquiere *et al.*, 1989; Henderson and Mello, 1990; Melander *et al.*, 1982). However, in these cases the conformers are in dynamic equilibrium and re-chromatography of either peak results in the appearance of two peaks (Gesquiere *et al.*, 1989). In contrast, the ovCNP-39A and ovCNP-39B isomers are homogeneous upon rechromatography. It is anticipated that this paradox will be resolved in future NMR experiments which are not feasible at present due to limited sample availability.

Natriuretic peptides in animal venoms

A recent report describing CNP-like immunoreactivity in *Bothrops jararaca* (South American pit viper) venom (Murayama *et al.*, 1997) is the only other example of a CNP in an animal venom, although *Dendroaspis angusticeps* (Green Mamba snake) venom contains an ANP-like peptide in low abundance (Schweitz *et al.*, 1992). Although the

endogenous function of CNP is unknown, the fact that CNP is released from endothelial cells following stimulation with cytokines suggests it may serve as an autocrine or paracrine mediator in cytokine-associated disorders (Suga *et al.*, 1993). Also, patients with septic shock and chronic renal failure show elevated serum CNP levels (Hama *et al.*, 1994). These findings, in addition to its discovery in *O. anatinus* and *Bothrops jararaca* venom, imply a pathophysiological function, a possibility which is dealt with below.

cGMP elevation in vascular smooth muscle cells

The structural and functional similarities between ovCNP-39 and CNP-22 suggest that they act at the same receptor. CNP potently stimulates cGMP accumulation in cultured VSMCs (Furuya *et al.*, 1990) through the activation of ANP_B receptors (Suga *et al.*, 1992). The lower levels of activity observed with ANP reflects the relatively low expression of ANP_A receptors in these cells (Suga *et al*, 1992). The present study therefore suggests that ovCNP-39 acts upon ANP_B receptors, with a comparable affinity to CNP-22. Furuya *et al.* (1992) demonstrated that only the disulphide-linked ring portion of CNP (CNP(6-22)) participates in the stimulation of cGMP accumulation in VSMCs. They found that the LKL motif (position 9-11 in CNP-22) was essential for this activity whereas substitution of Met for Gln at position 17 did not reduce activity. This is consistent with the observation that ovCNP-39 is equipotent with CNP-22, despite being extended at the N-terminus and having a Met→Thr substitution.

An increase in vascular smooth muscle cGMP, resulting from natriuretic peptide stimulation, produces vasodilatation (Wright *et al.*, 1996). CNP, while being less potent than ANP in eliciting vasodilatation in systemic arteries, produces relaxation in peripheral veins more potently than ANP through the activation of ANP_B receptors (Wei *et al.*, 1993), suggesting ovCNP-39 would act similarly. ovCNP-39-mediated

histamine release from mast cells may also promote vasodilatation and increase vascular permeability (Kuby, 1992). Both effects may serve to facilitate diffusion of venom components. These findings also indicate the likely contribution of ovCNP-39 to the hypotension and peripheral vasodilatation which accompanies the administration of *Oa*V to experimental animals (Kellaway and LeMessurier, 1935).

Uterus relaxing activity

The uterus relaxing activities of ovCNP-39 and CNP-22 may reflect the ability of these peptides to stimulate cGMP production in uterine tissue *via* ANP_B receptors (Dos Reis *et al.*, 1995). An increase in smooth muscle cGMP activates cGMP-dependent protein kinase (cGK) which is known to phosphorylate a number of proteins involved in smooth muscle relaxation (Archer *et al.*, 1994; Cornwell *et al.*, 1991; Komalavilas and Lincoln, 1994; Walsh *et al.*, 1995). The greater potency of CNP compared with ANP can be explained by the fact that ANP_A receptors, through which ANP acts, are localised mainly to the non-contractile endometrium. However, ANP_B receptors, through which CNP acts, are found on both the myometrial smooth muscle and the endometrium (Dos Reis *et al.*, 1995). In addition, ANP_B mRNA levels are about 100 times higher than those of ANP_A in the rat uterus (Dos Reis *et al.*, 1995). Yet surprisingly, ANP is more potent than CNP in stimulating cGMP production in segments of whole uterus (Dos Reis *et al.*, 1995). This apparent anomaly remains unresolved but it raises the possibility that relaxation of the rat uterus by CNP involves second messengers other than cGMP. Alternatively, diethylstilboesterol treatment may have modulated the activity of ANP receptors in the present study.

ovCNP-39 was found to relax the rat uterus more potently than either CNP-22 or ovCNP-39(18-39). Since the latter two peptides are equipotent and predominantly differ from ovCNP-39 by the absence of an N-terminal extension (Table 4.1), these

results suggest that the N-terminal extension underlies the increased potency of ovCNP-39. This is somewhat surprising considering that extensions of, or amino acid substitutions at, the N-terminus of CNP-22 do not appear to affect its interaction with ANP_B receptors in vascular smooth muscle (Furuya *et al.*, 1992) and raises the possibility of tissue heterogeneity for this receptor population. Alternatively, the N-terminal extension of ovCNP-39 may confer a higher affinity for other natriuretic peptide receptors such as ANP_A and ANP_C. ANP_C, which binds all natriuretic peptides with high affinity, undergoes endocytosis following binding and fuses with lysosomes where the peptide undergoes hydrolysis (Barr *et al.*, 1996). It thus appears to play a role in the control of circulating natriuretic peptide concentrations (Brandt *et al.*, 1995). However, studies using the specific ANP_C agonist, ANP(4-23)-NH_2 (cANP), have demonstrated that ANP_C receptors inhibit adenylate cyclase activity (Anand Strivastava *et al.*, 1990) and evoked catecholamine release in PC12 cells (Trachte *et al.*, 1995), but activate phospholipase C in vascular smooth muscle cells (Resink *et al.*, 1988) and Ca^{2+} channels in adrenal glomerulosa cells (Isales *et al.*, 1992). Whether any of these processes underlie the responses observed in the present study is unclear but their potential involvement could be investigated by studying the effect of cANF on uterine motility in the presence and absence of natriuretic peptides.

Oedemagenic and mast cell histamine releasing activities

It has previously been demonstrated that *Oa*V produces a marked paw oedema within 30 mins of injection, which is attenuated by the 5-HT_2 receptor-specific antagonist, ketanserin (de Plater, Hons. Thesis, ANU, 1993). This study has shown that both 100 μM ovCNP-39 and CNP-22 caused a significant paw oedema within 20 min of injection. The rapidity of the responses is consistent with the effects of mast cell degranulation rather than those arising from secondary mediators derived from

arachidonic acid metabolism (Bonta *et al.*, 1979). Therefore, the ability of various forms of the natriuretic peptides to degranulate rat peritoneal mast cells (that dominantly release histamine rather than 5-HT, which is released by cutaneous mast cells) was studied. All natriuretic peptides tested induced significant histamine release and were equipotent in this respect. However, the results suggest that ovCNP-39 and CNP-22 have a lower efficacy than α-ANP(1-28). The significance of such a difference in efficacy is unclear, as the mechanism of non-immunogenic[14] histamine release remains unresolved (see Cross *et al.*, 1995).

A number of polybasic peptides, including mastoparan (Higashijima *et al.*, 1988), substance P (Mousli *et al.*, 1990) and kinins (Bueb *et al.*, 1990), elicit histamine release from mast cells. Numerous structure-activity and binding studies suggest that polybasic peptides do not interact with conventional peptide receptors on mast cells (reviewed by Mousli *et al.*, 1990 and Mousli *et al.*, 1994), but rather produce exocytosis by directly activating the G-protein, G_{i3} (Aridor *et al.*, 1993). This is supported by the finding that a range of polybasic peptides stimulate the GTPase activity of purified G_i-proteins reconstituted into phospholipid vesicles (Higashijima *et al.*, 1990). The structural requirements for this activity include a minimum length, net positive charge, and an amphipathic α-helical structure (Higashijima *et al.*, 1990). These peptides are thought to insert into the bilayer such that their positive charges make direct contact with the G-protein (Higashijima *et al.*, 1990). Thus, it would be expected that such structural requirements would be important in determining histamine-releasing activity. However, Cross *et al.* (1995) demonstrated that mast cell degranulation by peptides is not necessarily dependent on the formation of amphipathic α-helices, and suggested that cationic secretogogues interact with negatively charged extracellular sites to

[14] immunogenic release in this case refers to release of histamine by IgE, attached to an allergen, and mediated through the IgE receptor.

promote subsequent activation of G_{i3}. These may be sialic acid residues, as treatment of mast cells with neuraminidase inhibits histamine release by peptides (Emadi-Khiav *et al.*, 1995).

Do ovCNP-39 and CNP-22 release histamine from mast cells in a similar, receptor-independent manner? This study indicates that the answer to this question is yes. Both peptides are polybasic and the results suggest that the N-terminal fragment of ovCNP-39, ovCNP-39(1-17) and the C-terminal fragment, ovCNP-39(18-39) release histamine with a similar potency. Interaction of CNP with ANP_B receptors minimally requires that the disulphide linkage is intact and that the stretch of amino acids, CNP-22(Leu^9-Lys^{10}-Leu^{11}) within the loop is present (Furuya *et al.*, 1992) Neither of these requirements are satisfied by the N-terminal fragment, ovCNP-39(1-17), suggesting that ANP_B receptors are not involved.

Conclusion

This chapter describes the purification and structure of a novel 39-residue C-type natriuretic peptide (ovCNP-39) from *Oa*V, which is responsible for the uterus relaxing activity of *Oa*V. The myorelaxant, cGMP-elevating, oedema-producing and mast cell degranulating activities of the C-type natriuretic peptide, ovCNP-39, from *Oa*V, have also been examined. The two *ex vivo* ovCNP-39 isoforms (which have identical amino acid sequences), synthetic ovCNP-39 and endogenous eutherian CNP-22, all produced a concentration-dependent relaxation of the rat uterus and cGMP elevation in VSMCs; ANP was significantly less potent in both respects. ovCNP-39 and CNP-22 produced oedema in the rat paw with a similar potency and their effective concentration range was comparable with that required for α-ANP-induced plasma extravasation in rat skin (Opgenorth *et al.*, 1990). An initial screen of HPLC fractions from a 10 kDa ultrafiltrate of the venom suggested that the two *ex vivo* isoforms of ovCNP-39 induce

histamine release from peritoneal mast cells. Further examination of this by constructing concentration-response curves for histamine release indicated that synthetic ovCNP-39 and fragments corresponding to the N- and C-termini, were equipotent with CNP-22 and ANP, although the latter exhibited greater efficacy.

The observation that ovCNP-39 elevates cGMP levels in vascular smooth muscle cells also suggests that it may underlie the vasodilatory and hypotensive effects of *Oa*V. Furthermore, the oedemagenic and mast cell histamine releasing activities of ovCNP-39 suggest that it may contribute to the local effects of envenomation.

CHAPTER 5

THE ELECTROPHYSIOLOGICAL EFFECTS OF *O. ANATINUS* VENOM ON RAT DORSAL ROOT GANGLION NEURONES

Introduction

The severe pain which accompanies *O. anatinus* envenomation may arise from the action of secondary mediators released from inflammatory cells. Alternatively, it may involve a primary, direct effect of a venom component(s) on nociceptive neurones or, indeed, a combination of both. The release of inflammatory mediators is likely to be at least partially involved. Chapter 4 describes a C-type natriuretic peptide, ovCNP-39, from the venom which elicits histamine release from mast cells. However, the longevity and severity of envenomation symptoms are not readily explained by such acute-phase effects which are moderate and short-lived by comparison (Keele and Armstrong, 1964). On the other hand, late-phase mediators, such as eicosanoids, cytokines (*e.g.* IL-1β) and nerve growth factor (NGF) have more prolonged algetic effects (Follenfant *et al.*, 1989; Ferriera *et al.*, 1988; Petty *et al.*, 1994) and may therefore be important contributors. However, this study addresses the possibility that the venom has a direct effect on nociceptors.

Electrophysiological studies of nociceptors *in vivo* have been hampered by the difficulty of gaining access to nociceptive terminals. Traditionally, extracellular recordings are made from isolated nociceptive fibres (C- and Aδ- fibres) which innervate a defined receptive field upon which algogenic agents are placed (*e.g.* Foster and Ramage, 1981) or injected into a neighbouring artery (*e.g.* Chahl and Iggo, 1977). The two major problems associated with these techniques are a) the amount of

electrophysiological information that can be obtained is limited as one has little electrical control over the fibres from which one is recording, *e.g.* voltage- and current-clamping is not possible and it is not possible to exchange intracellular ions; and b) it is difficult to determine whether the observed effects are produced or modulated by inflammatory mediators released by the algogen. These problems have been circumvented to an extent by the identification of a population of dissociated sensory neuronal cell bodies which express properties associated with nociceptors *in vivo*. Such properties include substance P-like immunoreactivity and responsiveness to the algogenic agents capsaicin, bradykinin and prostaglandin E_2 (PGE_2) (Baccaglini and Hogan, 1983). In addition, slowly conducting Aδ- and C-fibers, which are predominantly nociceptive, have small-medium diameter parent cell bodies (Harper and Lawson, 1985). Dorsal root ganglion (DRG) neuronal cell bodies which exhibit these properties are thus likely to be nociceptive and have been used to study the electrophysiological properties of nociceptors *in vitro* (Gold *et al.*, 1996a). In these neurones, capsaicin has been shown to activate a nonselective cation channel (Oh *et al.*, 1996) while prostaglandin E_2 and other algogenic agents, such as adenosine and 5-HT potentiate a tetrodotoxin-insensitive Na^+ current (Gold *et al.*, 1996b; Cardenas et al., 1997).

Using the whole-cell patch clamp technique, the effects of *Oa*V on small-to-medium diameter dorsal root ganglion neurones, many of which are likely to be nociceptors, have been investigated.

Methods

Venom preparation

Neat *Oa*V was suspended at a concentration of 1 mg ml^{-1} in either MES solution (mM: 135 NaCl, 3 KCl, 0.6 $MgCl_2$, 2.5 $CaCl_2$, 1.2 $NaHCO_3$, 10 glucose, 10 MES, titrated to

pH 6.1 with HCl, osmolarity adjusted to 320 mOsm using sorbitol) or HEPES, pH 7.4, solution (as above except HEPES replacing MES).

Cell culture

Male Wistar rats (5-8 weeks old) were anaesthetised with halothane (4% in O_2) and decapitated. A single incision was made in dorsal skin, the skin flaps pulled to either side and the vertebral column was cut at the cervical and lower lumbar regions. While holding the cervical end with forceps, the ribs were cut as closely as possible to the spinal vertebrae without damaging them and the column freed from the underlying mesentery. The column was placed in HEPES solution (described above), trimmed of excess tissue and remaining rib fragments, and dorso-ventrally hemisected. Dorsal root ganglia (DRGs) from both thoracic and lumbar regions were removed under a dissecting microscope using fine forceps and placed in H-16 Dulbecco's modified Eagle's medium (DMEM, supplemented with 100 units ml^{-1} penicillin G and 0.1 mg ml^{-1} streptomycin), containing 2.5 U ml^{-1} collagenase (Sigma, Type I) at 36 °C for 45 min. DRGs were then washed several times with DMEM and incubated in 5 mg ml^{-1} 'dispase'™ (Boehringer Manheim, Grade II, >0.5 $U.ml^{-1}$) at 36 °C for 30 min. DRGs were then resuspended in DMEM supplemented with 10 % fetal calf serum and the cell bodies were dissociated by trituration with a fire-polished pasteur pipette. DRG neurones were distributed into 10 wells of a 24-well tissue culture plate containing coverslips coated with collagen and poly-D-lysine (Sigma). Electrophysiological recordings were carried out 6 - 24 hours after plating.

Electrophysiology

Whole cell currents in DRG neurones were measured using an Axopatch 1D (Axon Instruments). Data were acquired on an IBM-PC compatible computer using pClamp software (Ver. 6, Axon Instruments) with a digitisation rate of 20 kHz and a MacIntosh

computer using Chart (Ver. 3.2, AD Instruments) software, with a digitisation rate of 2 Hz. Digitisation was accomplished using a TL-1 DMA interface (Axon Instruments) and a MacLab^{TM} digital/analog interface (AD Intruments), respectively. Electrodes were fabricated from Vitrex Modulohm I/S borosilicate glass using a Sutter Instruments (Model P-87) electrode puller. Electrode resistances ranged from 1.5-3 MΩ. Both capacitance and series resistance compensation (set at 80-90%) were used. Patch electrodes were filled with (mM) 147 KCl, 2 2Na-ATP, 0.5 2Na-GTP, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, titrated to pH 7.2 with KOH and osmolarity adjusted to 305 mOsm using sorbitol. The standard HEPES bath solution was as described above and the bath was continually perfused at a flow rate of approx. 1-2 ml min^{-1}. In some experiments, intracellular Cl^- was largely replaced with gluconate, giving a $[Cl^-]_i$ of 8.4 mM; alternatively, extracellular Cl^- was largely replaced with gluconate to give a $[Cl^-]_o$ of 9.2 mM; and to block K(Ca) currents, intracellular K^+ was completely replaced with TEA. The liquid junction potentials of these solutions were calculated (Barry, 1994) using a junction potential calculator program (JPCalcW © 1996 Peter H Barry, Axon Instruments).

O. anatinus venom (1 mg ml^{-1}) was applied to individual neurones by pressure ejection (5-10 psi) using a Picospritzer (GV Corp.) through flow pipes with internal diameters of 100 μm (GC Microbore DB-17, J & W Scientific). Flow pipes were attached to fine plastic tubing which was back-filled with 100-300 μl volumes of test solution and a three way tap was used to prevent front-filling with bath solution by capillary action. Very small amounts of material could be used in this way. The optimum position of flow pipes for superfusing cells was estimated visually by ejecting high-salt solution through the flow pipe into the bath filled with H_2O and monitoring solution flow under a phase-contrast microscope. Up to 5 flow pipes were glued

together and each could be moved into optimal position during an experiment, using a micromanipulator, to exchange solutions.

Solutions

Capsaicin (Fluka Chemica) solutions were made up freshly on the day of each experiment from a 10 mM stock in ethanol (stored at -20 °C), such that the final concentration of 1 μM capsaicin contained 0.01% ethanol. Stocks of thapsigargin (Research Biochemicals International) and 5-nitro 2-(3-phenylpropylamino) benzoic acid (NPPB, Research Biochemicals International) were also made up freshly in DMSO on the day of each experiment. These stocks were diluted to give a final concentrations of 1 μM thapsigargin and 10 μM NPPB each of which contained 0.02% DMSO.

High-performance liquid chromatography and SDS-PAGE

See Chapter 2 (General Materials and Methods) for details.

Data analysis and statistics

To compare *Oa*V currents under different experimental conditions, the average current was calculated. The inward current (I) resulting from each application was integrated with respect to time (t) and divided by the duration (T) of the response, *i.e.* Response X $= 1/T \int_0^T I\,dt$. Leak current was not subtracted. In some experiments, *Oa*V was applied twice; once in the absence and once in the presence of a particular inhibitor. The second response (Response B) was divided by the first response (Response A) to yield a response ratio (A/B). This was compared to the response ratio obtained by two applications of *Oa*V, both under normal conditions (*i.e.* in the absence of inhibitors). Results are expressed as means ± S.E.M. and compared using an unpaired Student's *t*-test. Significant differences were accepted at the $p < 0.05$ level.

Results

OaV - activated currents

Of 96 DRG neurones subjected to a 10 s application of 1 mg ml^{-1} *O. anatinus* venom at pH 6.1, 69 (72%) responded, following an average latency of 23.3 ± 3.0 sec, with an inward current characterised by multiple transient events (Fig. 5.1A). The current had a mean peak amplitude of -4.65 ± 0.34 nA which recovered to baseline in 56/69 neurones. In these cells, the mean duration of the response was 246.7 ± 36.5 s. 27/96 (28%) neurones were deemed to be unresponsive in that they exhibited effects similar to pH 6.1 alone, *i.e.* either a single transient inward current or no response (Fig. 5.1B). 1 mg ml^{-1} BSA in MES buffer (pH 6.1) also had the same effect as pH 6.1 alone. *Oa*V diluted in standard bath solution (HEPES, pH 7.4) produced either no effect or small, transient inward currents (n=5. Fig. 5.1C) which differed markedly from the more pronounced currents resulting from *Oa*V (MES) buffered at pH 6.1 (Fig. 5.1A, B). As the response was much more robust and reproducible at pH 6.1 these conditions were employed for subsequent experiments.

Correlation with capsaicin sensitivity

There was no correlation between sensitivity to capsaicin and *Oa*V (neurones were deemed to be capsaicin-sensitive if they responded to a 10 s application of 1 μM capsaicin with a characteristic inward current, Fig 5.1B). Of 6 capsaicin-sensitive neurones, 4 responded to *Oa*V and of 4 capsaicin-insensitive neurones, 3 responded to *Oa*V. As both capsaicin-sensitive and insensitive neurones responded to the venom DRG neuronal subtypes were discriminated, in subsequent experiments, solely on the basis of soma diameter (20-40 μm).

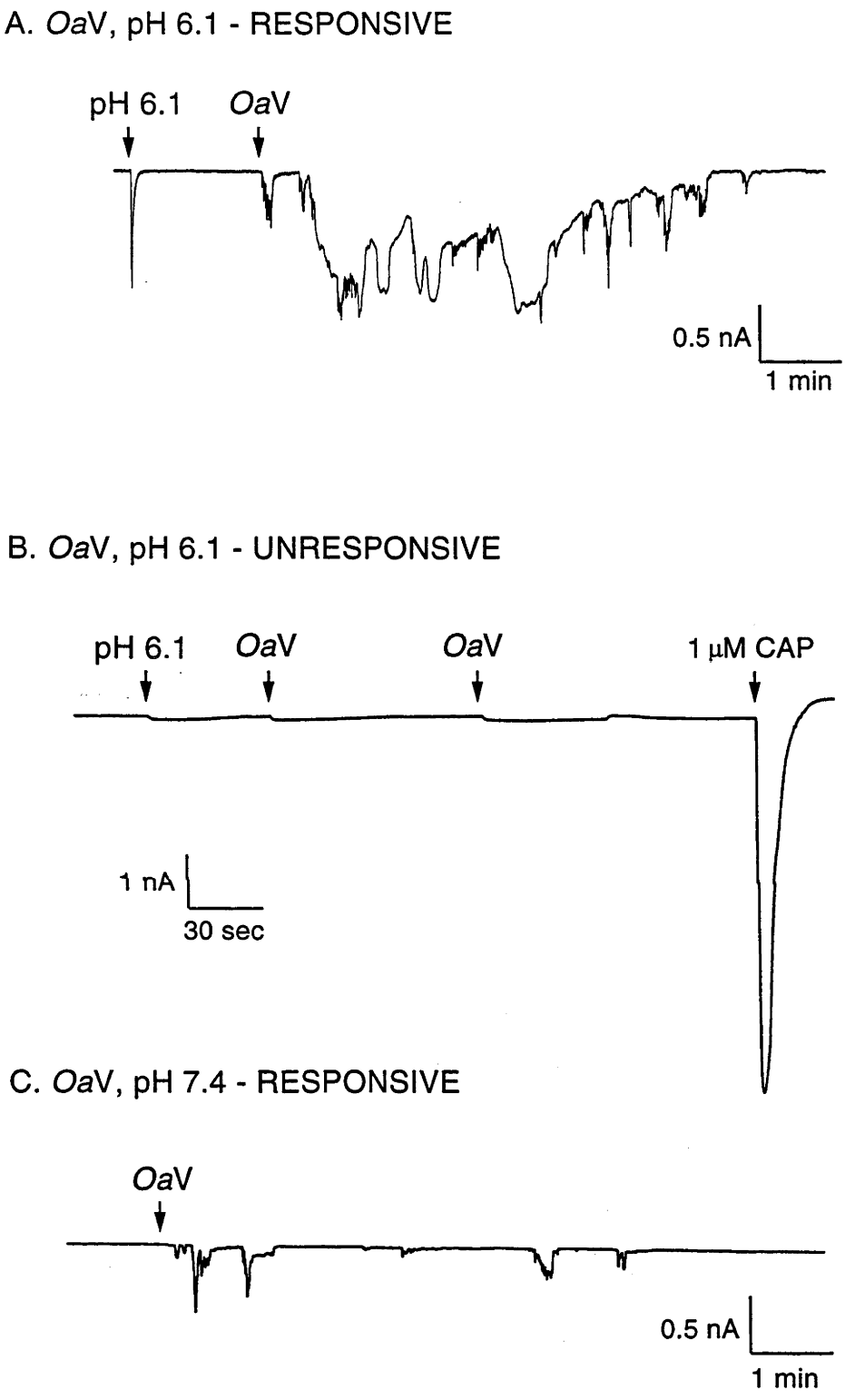


FIG 5.1. Typical responses of DRG neurones to *Oa*V. **A.** Effect of MES (pH 6.1) and 1 mg ml^{-1} *Oa*V (at pH 6.1), on a responsive DRG neurone. **B.** A neurone classified as unresponsive: this cell was also relatively insensitive to pH 6.1 but responded to a 10 s application of 1 μM capsaicin. **C.** The effect of 1 mg ml^{-1} *Oa*V made up at pH 7.4 in HEPES buffer. All applications were of 10 s duration.

Ionic basis and Ca^{2+} - dependence of the current

Current-voltage curves were obtained by applying a voltage ramp from -110 to 60 mV at a rate of 65 mV s^{-1}, before and during the response to *Oa*V (Fig. 5.2A). This rate was chosen as it was slow enough to remove voltage-dependent Na^+ currents by inactivation, but rapid enough so that the large transient events produced by *Oa*V did not produce disturbances in the resulting ramp current. Subtraction of the curve obtained before from that obtained during the *Oa*V response (Fig. 5.2B), yielded a linear inward current-voltage relationship in the -100 to -50 mV range (Fig. 5.2C). At membrane potentials more positive than -50 mV, voltage-dependent currents were activated, both in controls and following the application of *Oa*V. The linear range was therefore extrapolated (r-values ranged from 0.967 to 0.999) to give a reversal potential for the *Oa*V-activated current of -11.4 ± 2.0 mV (n = 22, Fig 5.2C and Table 5.1). This was close both to the calculated (using the Nernst equation) E_{Cl} of -6.9 mV and the reversal potential for the non-selective cation current (Currie and Scott, 1992; Crawford *et al.*, 1997a), suggesting the involvement of chloride and/or nonselective cation currents.

Ca^{2+}-dependent non-selective cation and chloride currents are known to occur in rat DRG neurones (Currie and Scott, 1992; Currie *et al.*, 1995). Therefore, to establish the dependence of the response to *Oa*V on intracellular Ca^{2+}, the Ca^{2+}-ATPase inhibitor, thapsigargin, was used to prevent the re-filling of intracellular Ca^{2+} stores. The effect of thapsigargin was investigated by applying *Oa*V twice in the presence of thapsigargin (Fig 5.3). As a control, two applications of *Oa*V were made in the presence of standard HEPES solution (n = 7) or standard solution containing 0.02% DMSO (n = 3). In control neurones, the second application of *Oa*V resulted in a larger ($p < 0.05$, n = 10) response ($1/T \int I.\partial t$, see Methods) than the first (Fig 5.3A, B). In contrast, thaspsigargin (1 μM) significantly ($p < 0.01$, n = 5) reduced the amplitude of

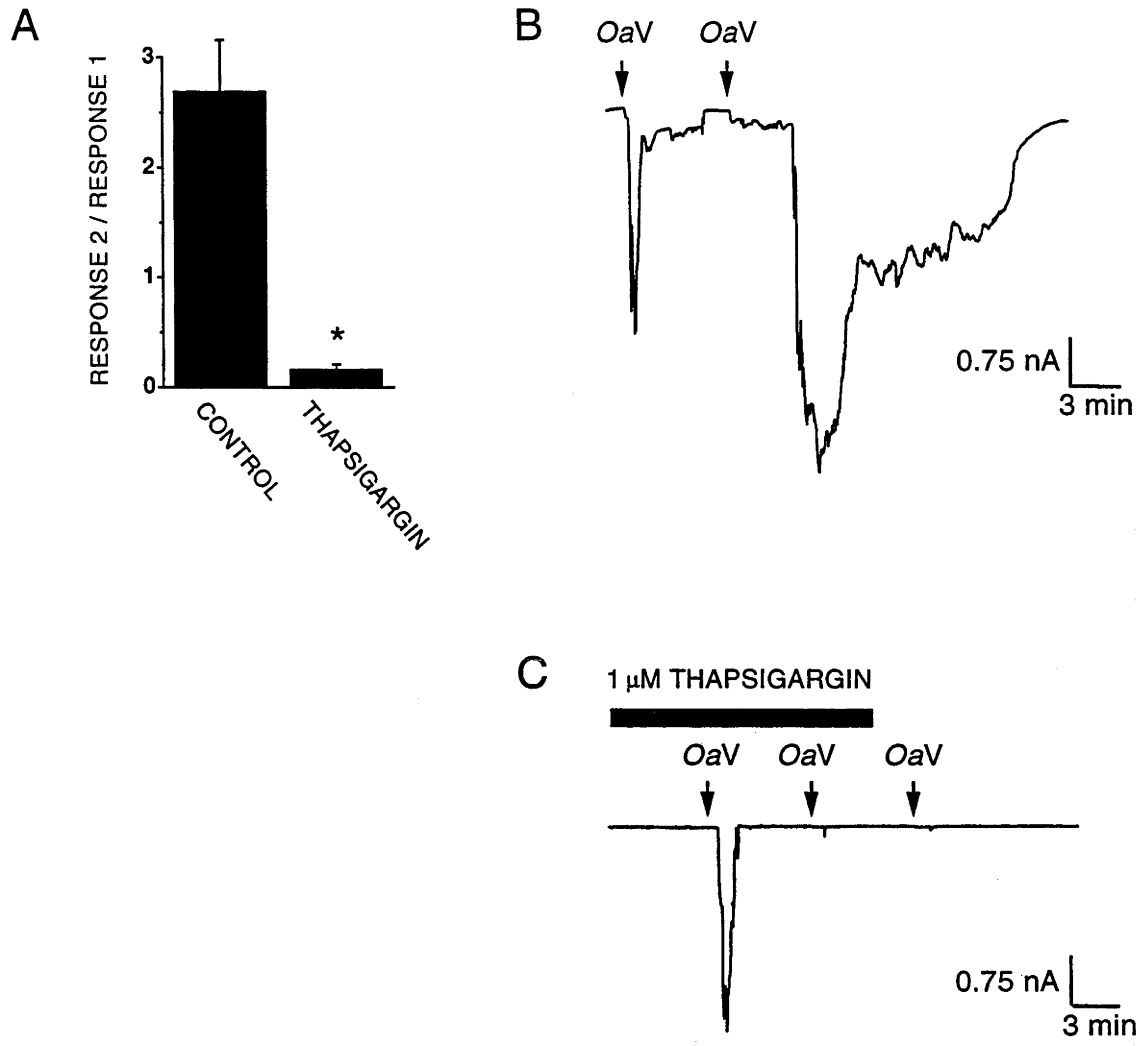


FIG. 5.3. The effect of thapsigargin on the ratio of the second to the first response to *Oa*V. *Oa*V was applied twice to each neurone and the response quantitated using the formula $1/d \int I.\partial t$. **A.** Ratio of response 2 / response 1 under control conditions (n = 10) and when 1 μM thapsigargin (n = 5) was included in the bath solution from the start of recording. **B.** and **C.** show examples of neurones responding to two applications of *Oa*V under control conditions (B) and in the presence of thapsigargin (C). In these experiments, *Oa*V (1 mg ml^{-1}) was applied for 10 s. * $p < 0.05$.

the second response compared to the first (Fig 5.3A, C) and this was irreversible upon washing out the thapsigargin (Fig 5.3C).

To establish which Ca^{2+}-dependent currents give rise to the *OaV* response, E_{rev} was determined following various ion replacements. Replacing extracellular Cl^- with gluconate resulted in a significant ($p<0.01$, $n=4$) positive shift in E_{rev} which, nevertheless, fell well short of the calculated E_{Cl} (61.6 mV) (Table 5.1). Replacing intracellular Cl^- with gluconate shifted E_{rev} in the opposite direction to that expected from the calculated E_{Cl} (Table 5.1). Any detected contribution from Cl^- would be reduced under these conditions because the linear part of the current-voltage curve used to obtain E_{rev} is centered around E_{Cl}, *i.e.* the driving force and, thus, Cl^- flux would be markedly decreased. However, no reduction in the *Oa*V-induced current was apparent. Thus these data suggest that the response to *Oa*V is probably dominated by a non-selective cation current.

When intracellular Cl^- was largely replaced with gluconate, neurones responded with a sustained current which lacked large transient events and failed to recover to baseline ($n = 5$, Fig. 5.4A). A similar effect was observed upon treatment with the Cl^- channel blocker, 5-nitro 2-(3-phenylpropylamino) benzoic acid (NPPB, 10 μM, $n = 3$, Fig. 5.4B). Thus Cl^- may underlie the transient events which characterise the *Oa*V-induced inward current.

Replacing intracellular K^+ with TEA resulted in a significant ($p<0.05$, $n = 6$) positive shift in E_{rev}, suggesting that a K^+ (possibly Ca^{2+}-dependent) current may also be activated by *Oa*V. However, its contribution is likely to be small given the E_{rev} observed under normal conditions of -11.4 ± 2.0 mV (Table 5.1) and the calculated E_K of -98 mV.

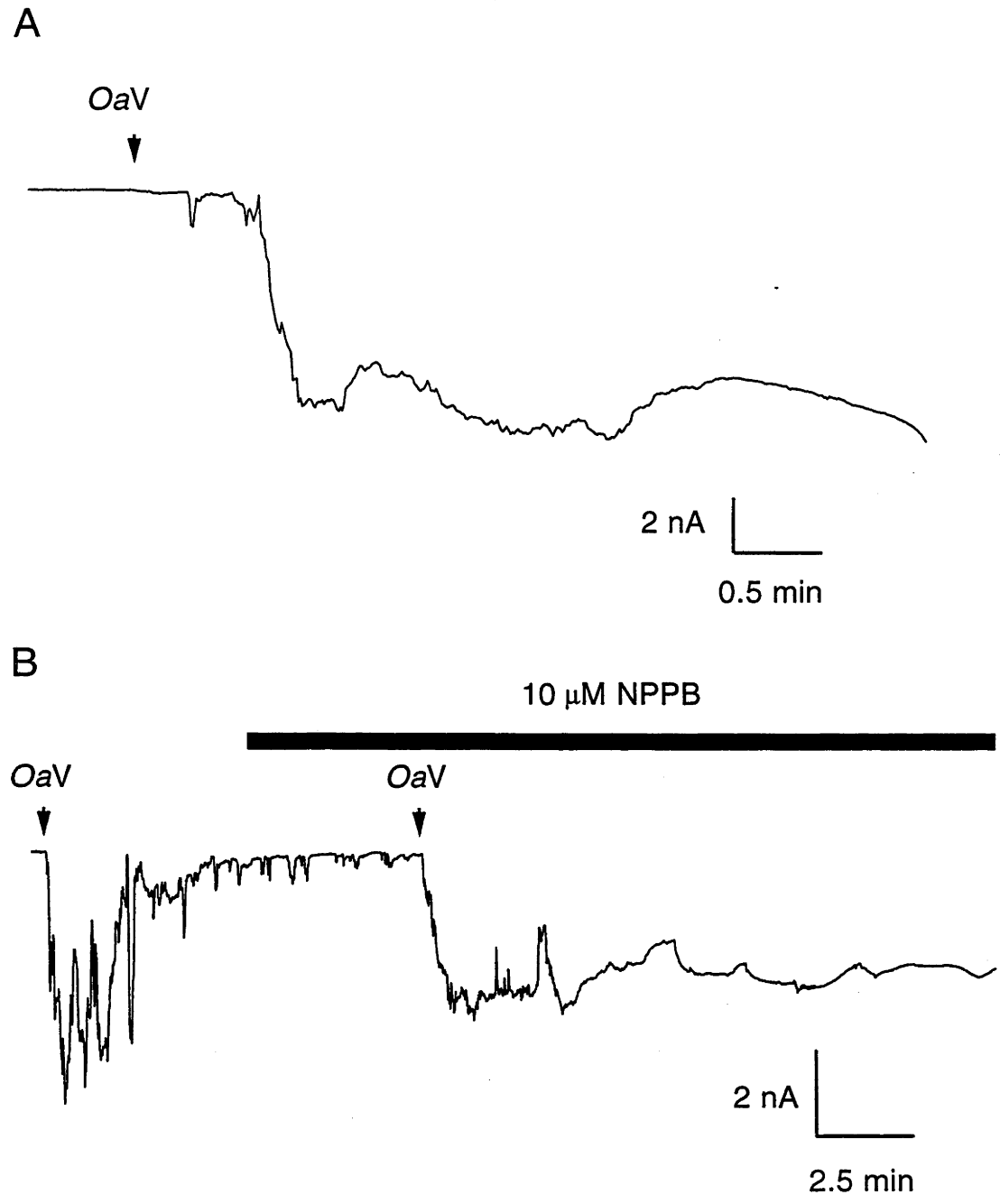


FIG 5.4. A. The effect of replacing intracellular Cl^- with gluconate on the response to *Oa*V (1 mg ml^{-1}, 10 s). **B.** A representative trace demonstrating the effects of the chloride channel blocker NPPB (10 μM, duration indicated by black bar) on the response to *Oa*V (1mg ml^{-1}, 10 s).

Table 5.1.

Cl^- equilibrium potentials, calculated using the Nernst equation, and observed reversal potentials following various ion substitutions. See Methods for solution compositions.

	N	E_{Cl} (mV)	E_{rev} (mV)
Standard solutions	22	-6.9	-11.4 ± 2.0
$[Cl^-]_o$ replaced with gluconate	4	61.6	$5.2 \pm 1.9^{**}$
$[Cl^-]_i$ replaced with gluconate	5	-70.7	$-1.1 \pm 3.5^{*}$
$[K^+]_i$ replaced with TEA	6	0.3	$0.0 \pm 2.8^{*}$

$^{*}p < 0.05$ $^{**}p < 0.01$

N-number of experiments; E_{Cl}-calculated Cl^- equilibrium potential; E_{rev}-mean extrapolated reversal potential from N experiments.

Purification and characterisation of the active material

Gel permeation HPLC (HP-GPC) was used to fractionate *Oa*V initially for the purpose of identifying factor(s) which give rise to the inward current activity. This approach had the advantage that the MES buffer (in which *Oa*V is most active) could be used as an eluent and fractions could be assayed directly without prior desalting or lyophilisation. HP-GPC of whole venom yielded 11 absorbance (280 nm) peaks, identified as GPC-I - GPC-XI (Fig 5.5A). Fraction GPC-V produced the inward current in DRG neurones characteristic of whole *Oa*V (Fig 5.6A).

This fraction was demonstrated to be heterogeneous by SDS-PAGE analysis (Fig 5.5E, Lane 3) and was subjected to further purification. Reversed phase HPLC (RP-HPLC) was used because this had previously been demonstrated to provide a good separation of low molecular weight *Oa*V proteins (see Chapter 3). RP-HPLC (μRPC C2/C18) fractionation of the active GPC fraction is shown in Fig 5.5B. Activity was associated with fraction III from this chromatogram (labelled RP-1III for reversed

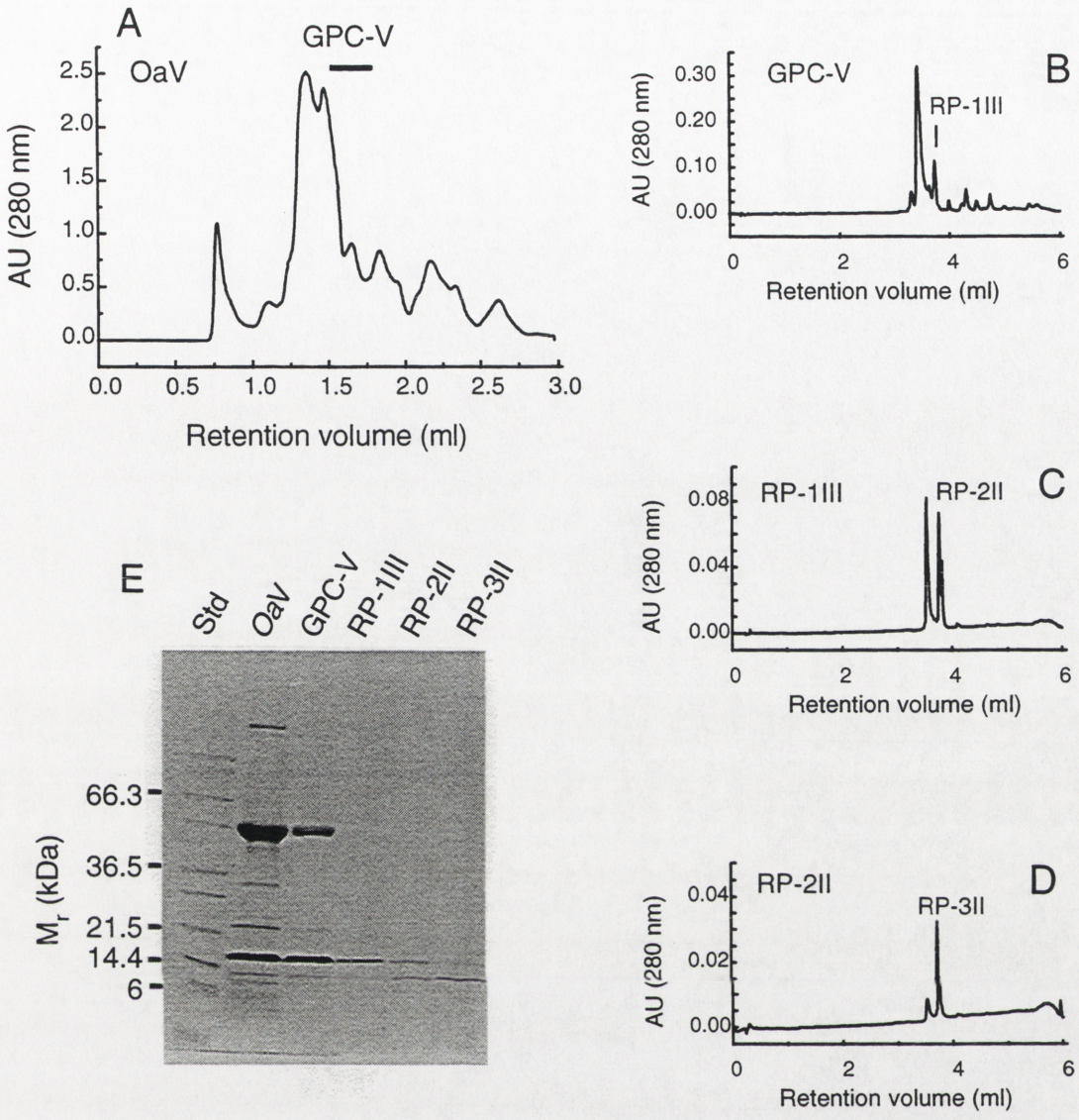


FIG 5.5. Purification of active material from *Oa*V. **A.** HP-GPC of *Oa*V. Activity was associated with GPC-V and this fraction was further purified by RP-HPLC. **B.** Activity was associated with fraction RP-1III. **C.** RP-1III was further purified by RP-HPLC, giving rise to an active fraction RP-2II which, **D.** was purified to near-homogeneity by a final RP-HPLC step (RP-3II). **E.** Tris-Glycine SDS-PAGE analysis of active fractions. Std = molecular weight standards.

phase I, fraction III). Again, this fraction was shown to contain several proteins (Fig 5.5E, Lane 4) and was therefore subjected to further rounds of RP-HPLC. A reduced flow-rate (100 μL min^{-1}) was used in these RP-HPLC steps to improve resolution (Fig 5.5C, D) and fractions were prepared between steps by dilution in 0.1% TFA/H_2O to give a final CH_3CN concentration of 5 %. Thus, lyophilisation, which tended to diminish the recovery of activity, was avoided. A 10 s application of 20 μg ml^{-1} of fraction RP-3II (resuspended in MES buffer, pH 6.1), resulting from the third round of RP-HPLC (Fig 5.5D), produced inward currents characteristic of whole venom (see Fig 5.6B) and yielded a comparable extrapolated E_{rev} of -5.28 ± 3.85 mV (n = 3). This fraction was not completely homogeneous by SDS-PAGE (Fig 5.5E, Lane 6). Although it was enriched in p12, the fraction also contained the 15 kDa protein, KLP, which was demonstrated to be inactive (n = 3) following purification by cation-exchange and reversed phase HPLC (see Fig. 3.5, Chapter 3 for details of purification).

Attempts were also made to purify the active material from *Oa*V using cation-exchange (CIEX) HPLC (Fig. 5.7A). As shown in Fig 5.6C, activity was associated with fraction II (CIEX FII, Fig. 5.7A) which exhibited marked heterogeneity by SDS-PAGE but also contained p12 (Fig. 5.7B). However, no activity was observed when p12 from CIEX FII was purified to apparent homogeneity by two subsequent RP-HPLC steps (Fig 5.7C, D, E and F). A shift in retention time was observed between the two RP-HPLC steps, *i.e.* p12 eluted somewhat later in the second step (Fig. 5.7E), raising the possibility that loss of activity was accompanied by a change in the conformation or association state of p12. A 15 kDa protein (KLP or OVL-15) was present in fractions I-IV from the second RP-HPLC step (Fig. 5.7E, F). These fractions were concentrated prior to SDS-PAGE (Fig. 5.7F) and the apparent absence of 15 kDa material from fraction I of the previous RP-HPLC step (Fig. 5.7C, D, Lane 3) reflects it relatively low abundance in this fraction.

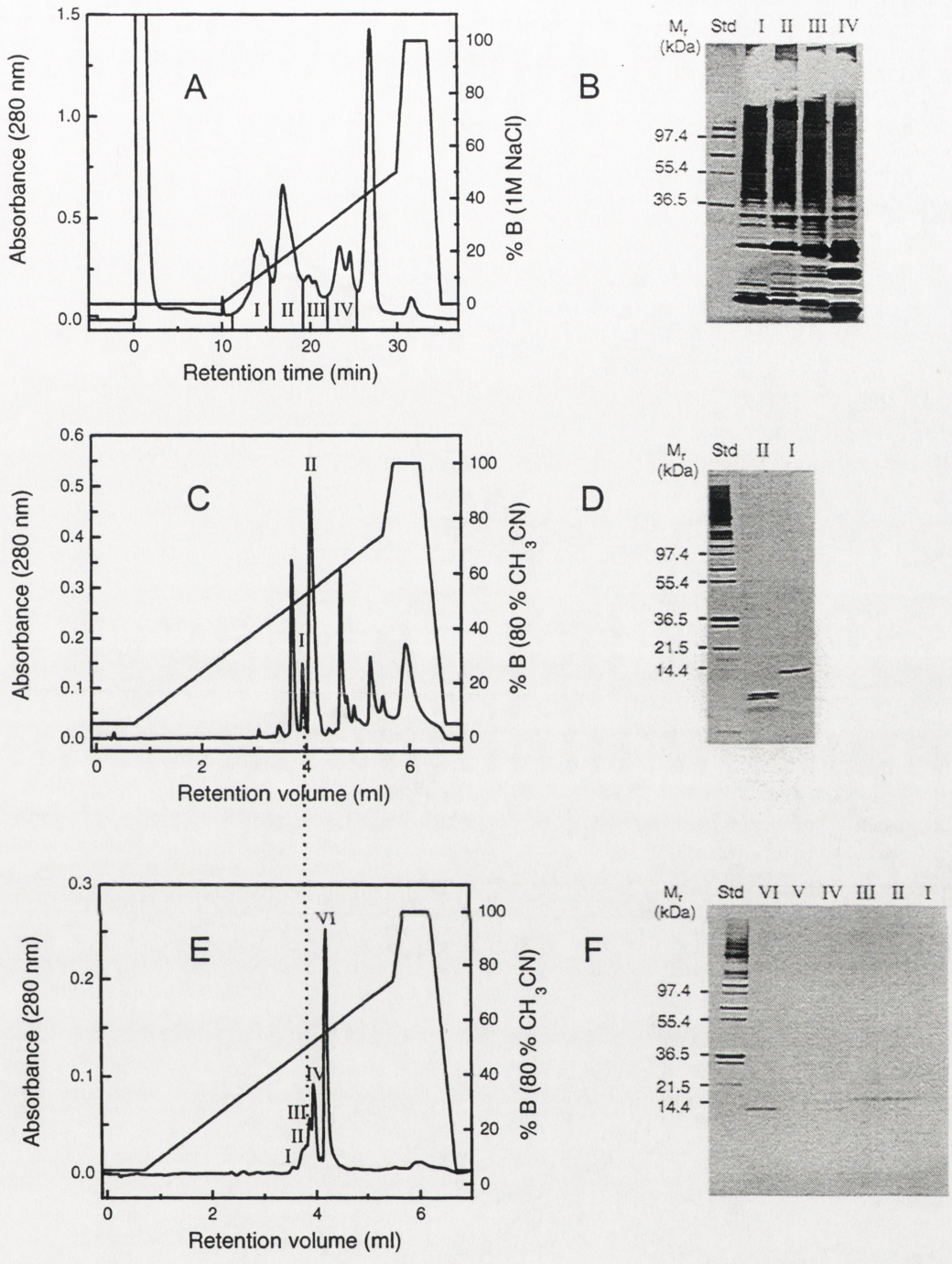


FIG. 5.7. Purification of p12 by cation-exchange and reversed phase HPLC. **A.** Cation-exchange HPLC of *Oa*V. Activity was associated with fraction II (CIEX FII). **B.** Tris-Glycine SDS-PAGE of cation-exchange fractions. **C.** RP-HPLC of CIEX FII. **D.** Tris-Tricine SDS-PAGE analysis of reversed phase fractions I and II from B. **E.** Further purification of p12 (I from C) by RP-HPLC. **F.** Tris-Tricine SDS-PAGE of RP-HPLC fractions from E. Note retention-volume shift between p12 - containing fractions I (C.) and VI (E.) indicated by the dotted line.

Active, homogeneous material was not recovered using the above purification strategies and a combination of anion-exchange HPLC and chromatofocusing was therefore used to avoid organic solvents which may have diminished the recovery of activity. The flow-through fraction from MonoQ was further fractionated using a MonoP chromatofocusing column (see Chapter 3), equilibrated with triethylamine buffer, pH 11.0 (Fig. 5.8A). A fraction, eluting at pH 10.1, was desalted by HP-GPC (Superose 12, Fig. 5.8B) and analysed by Tris-Tricine SDS-PAGE (Fig. 5.8C). A 10 s application of this fraction (80 $\mu g\ ml^{-1}$ in MES, pH 6.1), containing both p12 and p50, was demonstrated to be weakly and inconsistently active (n = 3, Fig. 5.8D).

From the different chromatographic dimensions employed, it is apparent that the protein common to each active fraction is p12. The active fraction RP-3II (Fig. 5.5) was subjected to N-terminal amino acid sequence analysis. Both a major and minor sequence was obtained. The minor sequence was identical to that of KLP (see chapter 3), which was the only contaminating protein detectable by SDS-PAGE (Fig 5.5E). The major protein sequence exhibited substantial homology with β-nerve growth factor (β-NGF). The protein was subjected to proteolytic digestion and the majority of the sequence determined from the resulting peptide fragments. p12 shares 90 % identity with β-NGF from mouse salivary glands and 67 % identity with NGF from *Naja naja* venom. Only two of the substitutions are at conserved sites (see Fig 6.5, Chapter 6).

The possibility that NGF from another source could produce the same effect as *Oa*V and p12-containing fractions was investigated using mouse salivary β-NGF (supplied by Prof. Ian Hendry, JCSMR and also obtained from ICN Pharmaceuticals Inc.). This material, at a concentration of up to 0.1 mg ml^{-1}, did not elicit *Oa*V-like inward currents (Fig. 5.9A, n=5). However, k-252a, which inhibits many of the biological activities of NGF (Knusel and Hefti, 1992) by binding to the cytoplasmic kinase domain of the high-affinity NGF receptor, trkA (Knight Jr *et al.*, 1997),

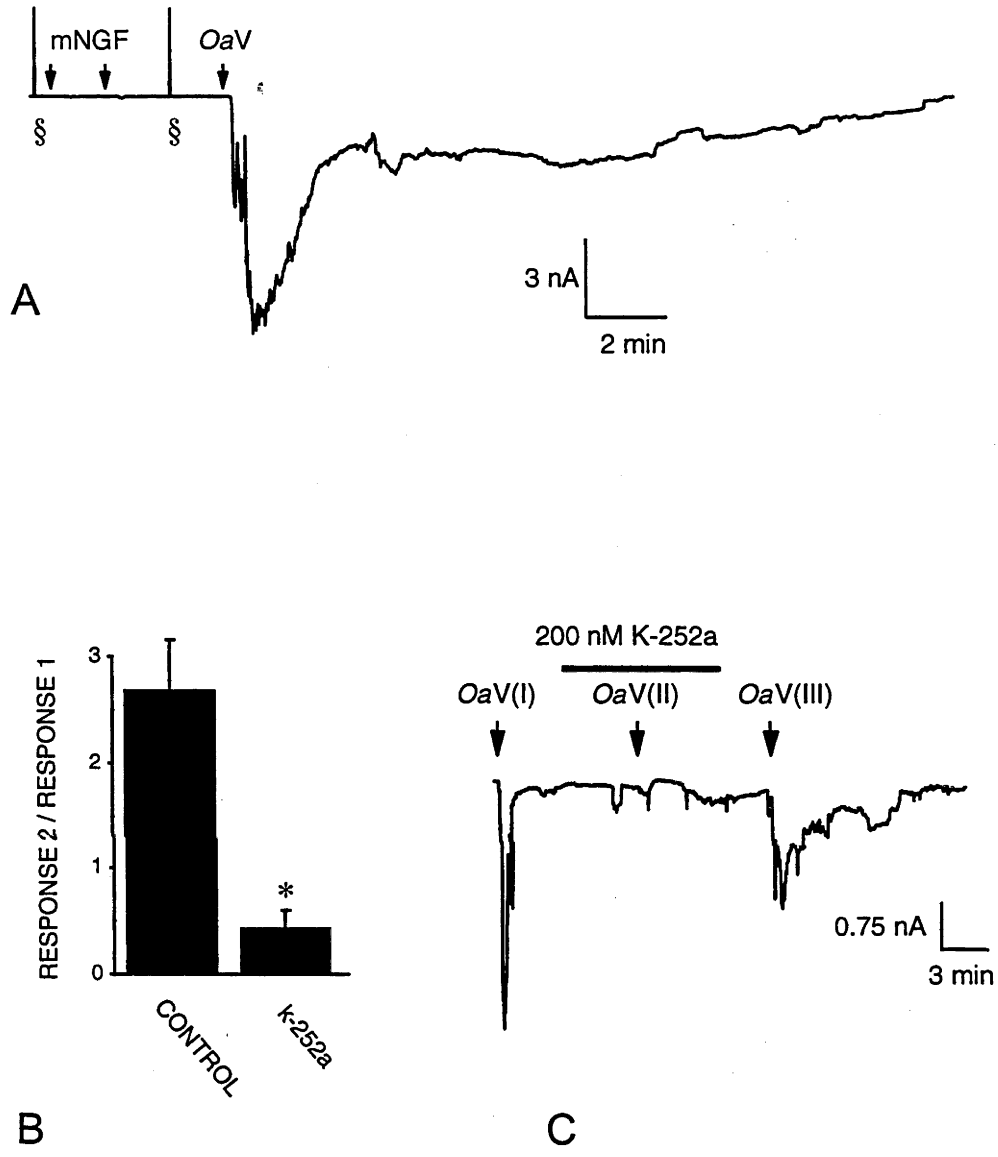


FIG 5.9. A. Effect of two 10 s applications of mouse NGF (mNGF, 0.1 mg ml^{-1}) in MES buffer (pH 6.1), followed by *Oa*V (1mg ml^{-1}). Artefacts (§) in current trace are due to applied voltage ramps. **B.** Effect of k-252a on the response to *Oa*V. Ratio of response 2 / response 1 under control conditions (n=10) and in the presence of 200 nM k-252a (n=4). *$p < 0.05$. **C.** Example of responses to *Oa*V in the absence and presence of k-252a.

significantly ($p<0.05$, $n = 4$) reduced the second response to *Oa*V relative to the first in two-application experiments (Fig 5.9B, C). This effect was at least partially reversible (Fig 5.9C) which is consistent with the reversibility of the TrkA-k252a interaction which has been demonstrated previously in radioligand binding studies (Knight Jr *et al.*, 1997).

Discussion

Although many venoms and toxins are considered potent algogens (Chahl and Kirk, 1975) and behavioural studies have demonstrated hyperalgesic effects of snake venoms (Teixeira *et al.*, 1994), this is the first example where the possibility of a direct effect of a venom on putative nociceptors has been investigated. *Oa*V was found to produce a lasting inward current in these neurones. Such an effect may contribute to the intractable pain which accompanies platypus envenomation.

While cultured DRG neurones provide a useful experimental model for nociceptive terminals, permitting the accessibility and electrical control not possible in terminals, the potential limitations of this model must be acknowledged. Firstly, it is assumed that the membrane properties expressed in DRG cell bodies are also expressed at the sensory terminals. The fact that capsaicin, bradykinin, protons and PGE_2 activate cell bodies as well as peripheral terminals (Baccaglini and Hogan, 1983; Gold *et al.*, 1996b; Bevan, 1996) and substance P and CGRP immunoreactivity is seen in both the soma and terminals (Gold *et al.*, 1996a), suggests the assumption is reasonable. However, other properties, such as the distribution of ion channels, may differ between the sensory terminal and cell body. For example, in central neurones, K^+ channel subtypes have specific cellular localisations (Hwang *et al.*, 1993). Further work needs to be done to determine whether this may be the case in DRG neurones.

There are also several factors which are believed to affect the cellular properties expressed by DRG neurones in culture. The age of the animal from which the DRGs are obtained is a critical factor, with properties such as the expression of capsaicin-sensitivity (Gold *et al.*, 1996a) and voltage-dependent Na^+ channels (Ogata and Tatebayashi, 1992) being age-dependent. In the present study, adult animals were used to prevent confounding results by age-dependent variations in the expression of nociceptor properties. However, the composition of the medium in which the DRG neurones are cultured also profoundly influences the expression of nociceptor properties. Withdrawal of NGF from the culture medium leads to a progressive loss of both proton and capsaicin sensitivity over several days which is reversible when NGF is added back to the medium (Bevan and Winter, 1995). Similarly, NGF removal leads to the disappearance of TTX-resistant action potentials (Aguayo *et al.*, 1991). NGF was not included in the medium used in the present study so NGF-dependent channels may have changed within the 24 h of culture. Nevertheless these neurones expressed both capsaicin and proton sensitivity and this may be attributed to the brevity of the acute NGF-free culture (loss of capsaicin and proton-sensitivity has a half time of several days (Bevan, 1996)). Other studies demonstrating the responsiveness of DRG neurones to PGE_2 and 5-HT, have involved acute culture in the presence of NGF (Gold *et al.*, 1996a and b). Thus, it is not clear whether NGF-treated or untreated neurones more accurately reflect the properties associated with nociceptive terminals. To what extent the absence of NGF in the medium affects the response to *Oa*V was not investigated in this study but could be resolved in future experiments by comparing the responsiveness of neurones to *Oa*V, cultured with or without NGF.

Comparison with other algogens

Several inflammatory mediators which are algogenic have been demonstrated to have a direct action on nociceptive neurones. However, currents produced by these agents appear to be quite different from those evoked by *Oa*V. Capsaicin activates an inward current in rat sensory neurones which inactivates rapidly upon removal of capsaicin (Petersen *et al.*, 1996). PGE_2, adenosine and 5-HT potentiate a TTX-resistant Na^+ current (Gold *et al.*, 1996b) whereas bradykinin activates a Ca^{2+} conductance (Burgess *et al.*, 1989) which declines within 30 s of its removal. Low pH, which produces pain and hyperalgesia in human subjects (Steen and Reeh, 1993) activates both transient and sustained (for the duration of low pH) nonselective cation currents in sensory neurones (Bevan, 1996). The extended time course and multiple transients which characterise the response to *Oa*V have not been observed following treatment with any of the above-mentioned algogens, although similar currents have been observed following application of glutamate agonists (Crawford *et al.*, 1997a), tachykinins and intracellular IP_3 (Li and Zhao, 1998).

Little correlation was observed between capsaicin sensitivity and *Oa*V sensitivity. This suggests that capsaicin and *Oa*V target different populations of sensory neurones. Capsaicin is considered to be a good marker for nociceptive neurones in that it causes pain (Torebjörk *et al.*, 1992), activates cutaneous nociceptors *in vivo* (Martin *et al.*, 1987) and is selective for small-medium diameter sensory neurones *in vitro* (Gold *et al.*, 1996a). However, it does not activate all nociceptors. 16 % of polymodal, 22 % of mechano-heat and 47 % of mechano-cold nociceptors are capsaicin-insensitive (Gold *et al.*, 1996a; Martin *et al.*, 1987). By comparison, 67 % of capsaicin-sensitive DRG neurones were activated by *Oa*V compared with 75% of capsaicin-insensitive neurones. Thus many nociceptive neurones which may not be activated by capsaicin are likely to be activated by *Oa*V.

pH-dependence

The response of DRG neurones to *Oa*V was pH-dependent in that robust and reproducible responses were elicited only when the venom was applied at pH 6.1 but not at pH 7.4. The physiological basis for this remains unknown. It has been demonstrated previously that the proton current elicited by rapidly lowering the extracellular pH to 6.1 is markedly potentiated in the presence of inflammatory mediators (Kress *et al.*, 1997), suggesting a synergy between these factors. Analogously, a component of the capsaicin-activated inward current, which normally exhibits marked tachphylaxis, is potentiated by subsequent applications of capsaicin at pH 6.3 (Petersen *et al.*, 1996). However, this may occur at the level of the capsaicin receptor as lowering the pH to 6.3 markedly potentiated the response of vallinoid receptor (VR1)-expressing oocytes to capsaicin (Caterina *et al.*, 1997).

Applying the venom at an acidic pH does have physiological implications. Inflammatory exudates are acidic (McCarty *et al.*, 1966) due to both a cellular switch to anaerobic glycolysis in inflamed tissue, thereby increasing production of H^+ (Kress and Reeh, 1996), and the active transport of lactic acid by leucocytes, into the interstitial space (McCarty *et al*, 1966). Thus the direct effect of *Oa*V upon nociceptive neurones may be potentiated markedly, *in vivo*, by tissue acidosis. Since the venom itself has a pH of 6.4 (see Chapter 3) this may also potentiate the activation of sensory neurones by *Oa*V.

Involvement of $[Ca^{2+}]_i$ and the ionic basis of the current

Thapsigargin prevents the refilling of intracellular Ca^{2+} stores by inhibiting sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pumps (Verkhratsky and Shmigol, 1996). Thus, its ability to inhibit inward currents induced by a second or subsequent application of *Oa*V suggests that the current is Ca^{2+}-dependent and that the

Ca^{2+} is derived from intracellular stores. It has been demonstrated previously that up to 10 min incubation with thapsigargin is required before Ca^{2+} from internal stores is depleted by resting leakage (Shmigol *et al.*, 1995a). Thus, inward currents resulting from the first *Oa*V application may result from the discharge of Ca^{2+} from stores not depleted by slow resting leakage during the relatively brief pre-incubation with thapsigargin.

These results should be interpreted with caution as it has been demonstrated that thapsigargin in the μM range also blocks high-voltage and, to a lesser extent, low-voltage activated Ca^{2+} currents in mouse DRG neurones (Shmigol *et al.*, 1995a) and adrenal glomerulosa cells (Rossier *et al.*, 1993). However, the observation that the second response to *Oa*V is reduced compared to the first supports the notion that thapsigargin inhibits the *Oa*V current by blocking Ca^{2+} release from intracellular stores. If the inhibition were dependent on the block of plasmalemmal channels, both responses should be affected as there would be no requirement for the prior depletion of Ca^{2+}.

Whether due to the block of SERCA pumps or plasmalemmal Ca^{2+} channels, the ability of thapsigargin to inhibit the *Oa*V-activated inward implicates its dependence on intracellular Ca^{2+} elevation. The reversal potential measured under standard conditions and following ion replacement also indicate the involvement of Ca^{2+}-activated nonselective cation currents (I_{CAN}) and possibly Ca^{2+} -dependent Cl^- currents ($I_{Cl(Ca)}$). $I_{Cl(Ca)}$ does not appear to play a major role as shifts in E_{rev} when gluconate replaced intracellular Cl^-, although significant, fell well short of the calculated E_{Cl}. Furthermore, NPPB, which has been demonstrated to block $I_{Cl(Ca)}$ tail currents in DRG neurones (Currie and Scott, 1992), did not block the response to *Oa*V. However, under these conditions, the currents were sustained and lacked large transient events suggesting that $I_{Cl(Ca)}$ may contribute to these events. Blocking K^+ currents by replacing intracellular

K^+ with TEA also resulted in a minor but significant shift in the observed E_{rev}, suggesting that a K^+ conductance, possibly $I_{K(Ca)}$, is also activated by *Oa*V.

Ca^{2+}-dependent currents, $I_{Cl(Ca)}$ and I_{CAN}, may be activated both by Ca^{2+} entering the cell through voltage-activated channels and caffeine-induced release of Ca^{2+} from intracellular stores (Currie and Scott, 1992; Crawford *et al.*, 1997a). These currents, which are also activated in DRG neurones by glutamate, cGMP (Crawford *et al.*, 1997a), and the sperm factor oscillin (Currie *et al.*, 1992; Parrington *et al.*, 1996) exhibit some of the characteristics of the *Oa*V-mediated response. These include a comparable delay to the onset of the response and pronounced transient events underlying the observed whole cell currents (Crawford *et al.*, 1997a). These similarities support the idea that release of Ca^{2+} from intracellular stores also gives rise to the *Oa*V response.

Two distinct mechanisms mediate Ca^{2+} release from intracellular stores in neurones: inositol (1, 4, 5)-trisphosphate (IP_3)-induced release and Ca^{2+}-induced release (CICR). These mechanisms involve two different types of Ca^{2+} release channel. The Ca^{2+} release channel implicated in CICR, which is characterised by specific binding of the plant alkaloid, ryanodine, is known as the ryanodine receptor (RYR). Several subtypes, arising from three distinct genes have been identified, and correspond to the skeletal (RYR1), cardiac (RYR2) and brain (RYR3) isoforms (Pozzan *et al.*, 1994; Meissner, 1994). The mechanistic basis of *Oa*V-induced release of Ca^{2+} has yet to be investigated. However, similar currents evoked in DRGs by glutamate and cGMP appear to be mediated by both IP_3R and RYR (Crawford *et al.*, 1997b) and may involve CICR (Crawford *et al.*, 1997a). In addition, caffeine, which also evokes similar currents (Currie and Scott, 1992), specifically lowers the threshold of CICR channels for Ca^{2+} ions enabling CICR at resting Ca^{2+} concentrations (Sitsapesan and Williams, 1990). The possible involvement of the RYR and CICR in the resonse to *Oa*V may be

addressed in future experiments by investigating the relative abilities of ryanodine and heparin (an inhibitor of the IP_3 receptor) to inhibit the *Oa*V-activated currents.

CICR gives rise to elevations in intracellular Ca^{2+} only in those DRG neurones which are caffeine-sensitive (Shmigol *et al.*, 1995b). Interestingly, this represents around 70 % of DRG neurones (Shmigol *et al.*, 1995b), which is equivalent to the number responding to *Oa*V in this study.

Purification of p12/NGF from OaV and its involvement in inward current activity

SDS-PAGE analysis revealed that the protein, p12, was common to each chromatographic fraction that elicited inward currents in DRG neurones and this was found to share substantial amino acid sequence homology with NGF. NGF produces hyperalgesia when injected into human subjects (Petty *et al.*, 1994) and experimental animals (Lewin *et al.*, 1993). It appears to act directly on sensory neurones but also releases autacoids from inflammatory cells and sympathetic neurones (Woolf *et al.*, 1996). Interestingly, acute administration of NGF to chick DRG neurones produces transient increases in intracellular calcium (Yamashita and Kawana, 1991); it rapidly prolongs the duration of the Ca^{2+}-dependent component of the action potential in mouse DRG neurones (Shen and Crain, 1994) and sensitises visceral Aδ- and C-fibre afferents (Dmitrieva and McMahon, 1996). NGF also evokes increases in cytosolic free Ca^{2+} in TrkA-expressing C6-2B glioma cells (De Bernardi *et al.*, 1996). Despite these effects, mouse salivary β-NGF did not evoke the inward currents characteristic of *Oa*V or p12-containing fractions.

There could be a number of reasons for the lack of activity of mouse NGF. Firstly, p12 may differ structurally from NGF or, secondly, a co-factor, present in the venom, may be required for activity. Regarding the first possibility, two peptide fragments, comprising residues 74-85 and the C-terminal 13 residues, have not been

recovered from the digests (see Chapter 6 for details) and may differ in sequence from the mouse form. NGF binds to two distinct receptors, one with high affinity, a tyrosine kinase receptor, TrkA, and one with low affinity, p75, which is a member of tumour-necrosis-factor-receptor superfamily (Smith *et al.*, 1990). Binding of NGF with p75 occurs *via* three positively charged residues, Lys-32, Lys-34 and Lys-95 (Ibanez, 1994). The corresponding Lys residues are present in p12, suggesting that its binding to p75 would be commensurate with NGF. Mutations to these residues do not appear to affect TrkA activation (Ibanez *et al.*, 1992). However, deletion of residues 112-118 in the C-terminus, prevents TrkA-mediated biological activities of NGF (Drinkwater *et al.*, 1993) and both His-75 and His-84 have been found to be important for the interaction of NGF with TrkA (Woo and Neet, 1996). As the corresponding sites containing these residues have not been sequenced in p12 they may differ and, accordingly, interaction with TrkA and biological activity may also differ. Either of the substitutions at conserved sites (*e.g.* Asn-25 or Lys-61) in p12, could also affect its biological activity although the corresponding residues in mouse NGF do not appear to be involved in the interaction with TrkA.

It is possible that the discrepancy between the ability of p12-containing fractions and mouse NGF to elicit inward currents could be due to the involvement of a co-factor. As p12 could not be purified to homogeneity, co-purified proteins or other factors could act synergistically with p12 to produce or to elevate the response. For example, larger inward currents were observed in fractions containing both p12 and KLP, compared with those containing p12 and other proteins and, therefore, a synergy between p12 and KLP may exist. KLP shares homology with members of Kunitz-type protease inhibitor-like family of proteins (see Chapter 3), which includes the β-amyloid protein implicated in Alzheimer disease. NGF potentiates the neurotoxicity of this

protein in hippocampal neuones (Yanker *et al.*, 1990) and it appears to contribute to the trophic effects of NGF in PC12 cells (Majocha *et al.*, 1994).

There are additional instances of synergistic effects between NGF and other factors. For example, the cytokines IL-1β and IL-6 increase NGF-mediated tyrosine phosphorylation and process outgrowth in olfactory neuroepithelial cells (Vawter *et al.*, 1996), laminin increases the trophic effects of NGF in sympathetic neurones from adult rats (Cowen *et al.*, 1997), and retinoic acid is required for the NGF induction of transin gene expression in PC12 cells (Cosgaya, 1997). It is therefore possible that a synergy exists between p12 and another protein or undetermined factor in the venom and that it is the absence of this factor which underlies the lack of inward current activity of mouse NGF. Clearly, it would be of use to screen all chromatographic fractions (not containing p12) in order to determine whether there is a factor which can confer this activity on mouse NGF.

The ability of the TrkA inhibitor, k-252a to attenuate the response to the venom (Fig. 5.9B) suggests the involvement of TrkA receptors. TrkA kinase activity leads to the phosphorylation of phospholipase Cγ (Kim *et al.*, 1991) and subsequent phosphatidylinositol hydrolysis to produce IP_3 and IP_3-induced release Ca^{2+} from intracellular stores (Berridge, 1993). Indeed, in C6-2B glioma cells expressing TrkA, k-252a blocked the NGF-evoked rise in intracellular Ca^{2+} (De Bernardi *et al.*, 1996).

Only 45 % of DRG neurones from adult rats express TrkA and this expression is largely confined to small-diameter neurones (Bennett *et al.*, 1996). This is consistent with the proportion of cells (72 %) responding to *Oa*V, given that small-medium diameter neurones were specifically targeted. However, it is possible that *Oa*V acts *via* a receptor other than TrkA, whose activity is also inhibited by k-252a. For example, k-252a is a potent inhibitor of protein kinase C (Gschwendt *et al.*, 1996) and the *Oa*V activity may involve a pathway which utilises this enzyme. The requirement of TrkA

for the response may be clarified in future experiments using specific antibodies to TrkA which prevent it from binding NGF.

Conclusion

A direct effect of *Oa*V on nociceptive neurones may be largely responsible for the intractable pain which accompanies platypus envenomation. The possibility of a venom or venom component having a direct action on nociceptive neurones apparently has not been investigated previously, despite the fact that numerous venoms and toxins are algogenic (Chahl and Kirk, 1975). This is probably due to the difficulty of unambiguously defining a direct as opposed to an indirect action of nociceptors in *in vivo* and *in situ*[15] preparations. The use of DRG neurones which express nociceptor properties largely circumvents this problem but relies on the assumption that the nociceptive DRG cell soma expresses the same properties as its terminal. The fact that other pain-producing substances generate inward currents in these neurones suggests that a direct action of *Oa*V on these neurones is also likely to produce pain.

The magnitude of the *Oa*V-mediated inward currents and the fact that they far outlast the duration of the application, suggests that *Oa*V may also be more potent in this respect than other algogens. Upon envenomation, nociceptive endings would almost certainly be exposed to higher concentrations of *Oa*V than used in this study (the venom was diluted around 40-fold to achieve the standard concentration used for this study) and for a longer time (*i.e.* greater than 10 sec). The conditions used to elicit the inward currents in this study are therefore, if anything, conservative in comparison to what might be expected following envenomation. Thus it is probable that if the effect of *Oa*V on DRG neuronal cell bodies truly reflects its activity at the nociceptive

[15]For example, the method of Steen and Reeh (1992) which utilises a superfused skin-saphenous nerve preparation, removed intact from the rat, that allows the application of chemicals to the corium side of identified receptive fields

terminal, this direct action would contribute substantially to the painful effects which accompany envenomation.

CHAPTER 6

THE NERVE GROWTH FACTOR (NGF) FROM *O. ANATINUS* VENOM

Introduction

Nerve growth factor (NGF) was originally described as a diffusible agent derived from mouse sarcoma cells which elicited neurite outgrowth in embryonic chick neurones (Levi-Montalcini and Hamburger, 1953). Attempts to purify the active factor proved to be difficult due to the continued presence of contaminating nucleic acids and this led to the use of crude snake venom (from *Agkistrodon piscivorus*) as a source of phosphodiesterase. It was found, serendipitously, that the snake venom actually produced more neurite proliferation than the sarcoma sample and this observation ultimately led to the purification and identification of nerve growth factor (NGF) from snake venoms (Cohen and Levi-Montalcini, 1956; Cohen, 1959). Subsequently, NGF was found to be abundant in male mouse submaxillary glands (Cohen, 1960) and NGF from this source has been extensively studied due to its abundance and relative ease of purification.

NGF was the first neurotrophic factor to be discovered and, in many ways, is considered the prototype neurotrophin[16] although three other mammalian neurotrophic factors have been identified: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Kullander *et al.*, 1997). In the developing peripheral nervous system, NGF plays an important role in the survival and maintenance of sympathetic neurones and a subpopulation of sensory neurones, which

[16] Neurotrophins are factors which promote the survival, differentiation, and maintenance of neurones

includes nociceptive neurones. Centrally it supports the survival and growth of basal forebrain cholinergic neurones in the brain (Crowley *et al.*, 1994).

NGF from mouse submaxillary glands exists as a high molecular weight complex of 140 kDa, with a sedimentation coefficient of 7S (Varon *et al.*, 1968). 7S NGF consists of 5 subunits, with a stochiometry of 2 α, 2 γ and 1 β. The β-subunit (β-NGF, M_r approx. 13.2 kDa) is the most extensively studied subunit and is responsible for the nerve growth-promoting activity of NGF. It is generally isolated as a non-covalently linked dimer, the 2.5S complex, but both the monomeric and dimeric forms are active (Stach and Shooter, 1974). The γ-subunit is a member of the kallikrein family of trypsin-like serine proteases and appears to play a key role in the processing of the β-NGF precursor at the C-terminus (Fahnestock, 1991). The sequence of the α-subunit shows significant homology with the γ-subunit and also appears to be a kallekrein although it lacks enzymatic activity and its function is unknown (Fahnestock, 1991). All snake venom NGFs are homologous with mouse β-NGF and, with the exception of that from *Crotalus adamanteus* venom, which associates with a γ-subunit (Perez-Polo *et al.*, 1978), are not found associated with other subunits. It has also been demonstrated that neither the mouse α- or γ- subunits bind to *Naja naja* venom NGF (Server *et al.*, 1976).

Kostiza and Meier (1996) have classified snake venom NGFs into the following four classes (Type I-IV) on the basis of structural characteristics. Type I NGFs are dimeric forms similar to mouse β-NGF which have a subunit molecular weight of aroung 13, 000. Examples of species whose venom contains Type I NGFs include the cottonmouth moccasin (*Agkistrodon piscivorus),* South American pit viper *(Bothrops jararaca)* and Taiwan cobra *(Naja naja atra).* Type II NGFs have a higher molecular weight and contain 10-20 % carbohydrate and may or may not exist as dimers. Type II

NGFs are found in the venoms of the common lancehead *(Bothrops atrox),* Malayan pit viper *(Agkistrodon rhodostoma),* Southern European sand viper *(Vipera ammodytes)* and Russell's viper (*Vipera russellii).* Type III NGFs have a homodimeric structure linked by disulphide bonds and are found in the venoms of the Formosan banded krait (*Bungarus multicinctus)* and puff adder (*Bitis arietans).* Type IV NGF refers to that found in diamondback rattlesnake *(Crotalus adamanteus)* venom which associates with a γ-subunit as described above. Snake venom and mammalian NGFs share about 64 % sequence homology and NGF from *Naja naja* and *Vipera russelli* venoms show a similar neurotrophic activity to mouse NGF (Koyama *et al.*, 1992; Kozista and Meier, 1996).

The probable contribution of *Oa*V NGF to the *Oa*V-induced inward current activity in dorsal root ganglion neurones has been described in Chapter 5. The fact that mouse NGF did not produce inward currents raised the possibility that *Oa*V NGF, whose primary structure elucidation is the subject of this chapter, may have a novel structure.

Methods

NGF Bioassay

A neuronal survival assay was used to assay the NGF activity of *Oa*V. Dissociated neuronal cultures from dorsal root ganglia (DRG) of 8-day old chick embryos, prepared by Prof. Ian Hendry and Katarina Haydon (Division of Neuroscience, John Curtin School of Medical Research), were used. DRGs were dissected from the embryos and incubated at 37 °C in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) containing 0.1 % trypsin for 20 min. DRGs were transferred into Dulbecco's modified Eagle's medium (DMEM) containing 2 % foetal calf serum, dissociated by trituration using a fire-polished Pasteur pipette and plated in the polyornithine and laminin-coated wells of a

96-well culture plate. The wells contained 10-fold serial dilutions of β-NGF or *Oa*V. Neuronal survival was quantified after 48 h by counting the number of bright-phase, spherical neurones occupying 4 microscope (phase-contrast) fields in each well. In the absence of NGF, very few neurones survive for 48 h under these conditions.

N-terminal Edman sequencing

Purified *Oa*V NGF was reduced, *S*-pyridylethylated (see Chapter 2) and subjected to direct N-terminal Edman sequencing following passive adsorption onto PVDF. The protein was also subjected to *in situ* digestion in excised Coomassie-stained gel pieces (see Chapter 2) using endoproteinase Lys-C (lysyl endoproteinase from *Achromobacter lyticus*, Wako), or in-solution digestion with chymotrypsin and endoproteinase Arg-C (both sequencing grade, Boehringer Manheim Biochemica). The resulting peptide fragments were purified by RP-HPLC (using a Sephasil C18 column, Pharmacia or a Brownlee Aquapore OD-300 column, Perkin-Elmer), adsorbed onto PVDF and submitted to Edman sequencing (see Chapter 2).

Database search protocols and algorithms

NGF sequences were obtained from the National Library of Medicine Genbank Protein Sequence database (URL: http://www.ncbi.nlm.nih.gov/PubMed/) and were aligned as a family using the Clustal W multiple sequence alignment program (Ver. 1.7), part of the BCM Search Launcher (URL: http://dot.imgen.bcm.tmc.edu:9331/multialign/multi-align.html).

Results

A preliminary investigation of the neurotrophic effects of *Oa*V revealed that whole *Oa*V promoted the survival of chick sensory ganglion neurones in a dose-dependent manner. Fig. 6.1 shows the effects of increasing *Oa*V and mouse β-NGF

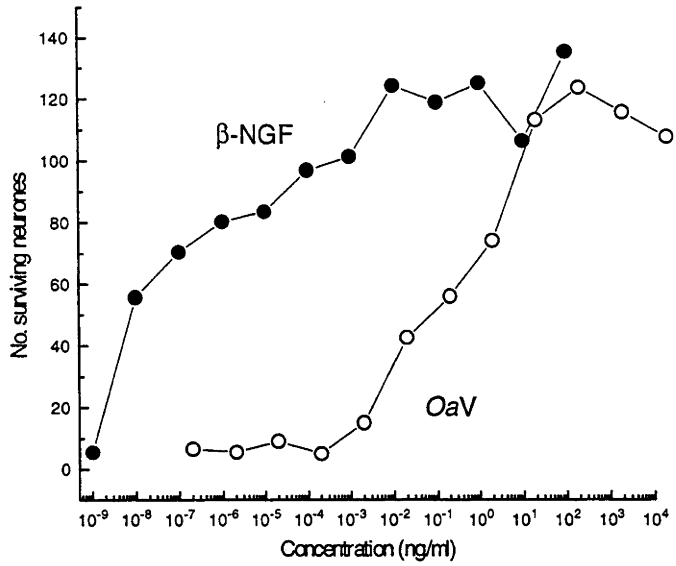


Fig. 6.1. Concentration-response of 8 day old chick dorsal root ganglion neurones to *O. anatinus* venom (OaV, open circles) and mouse β-NGF (closed circles). The number of surviving neurones at each concentration was determined after 48 hr by counting the number of bright- phase cells in 4 microscope fields. Each point represents the mean of duplicate experiments.

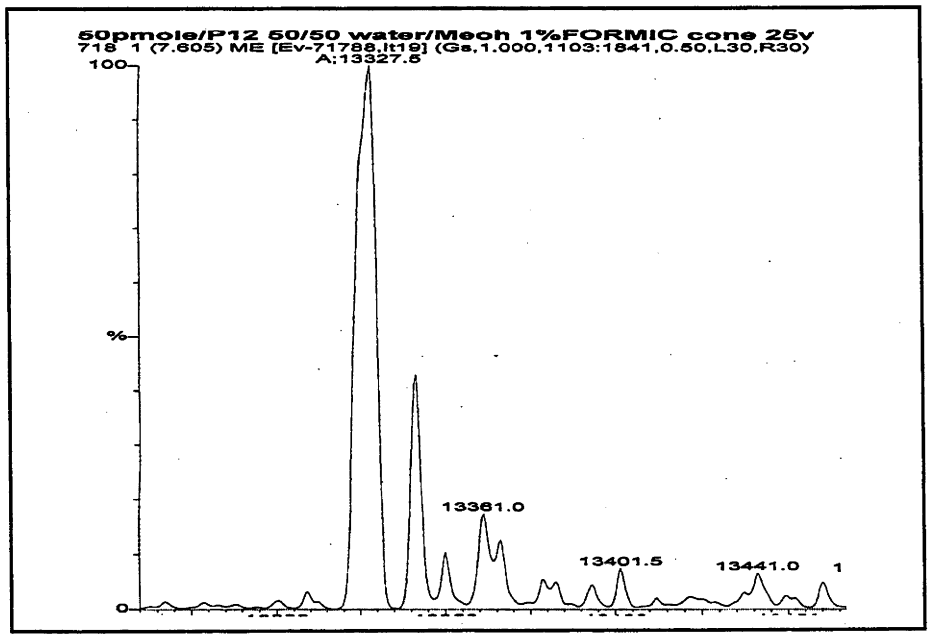


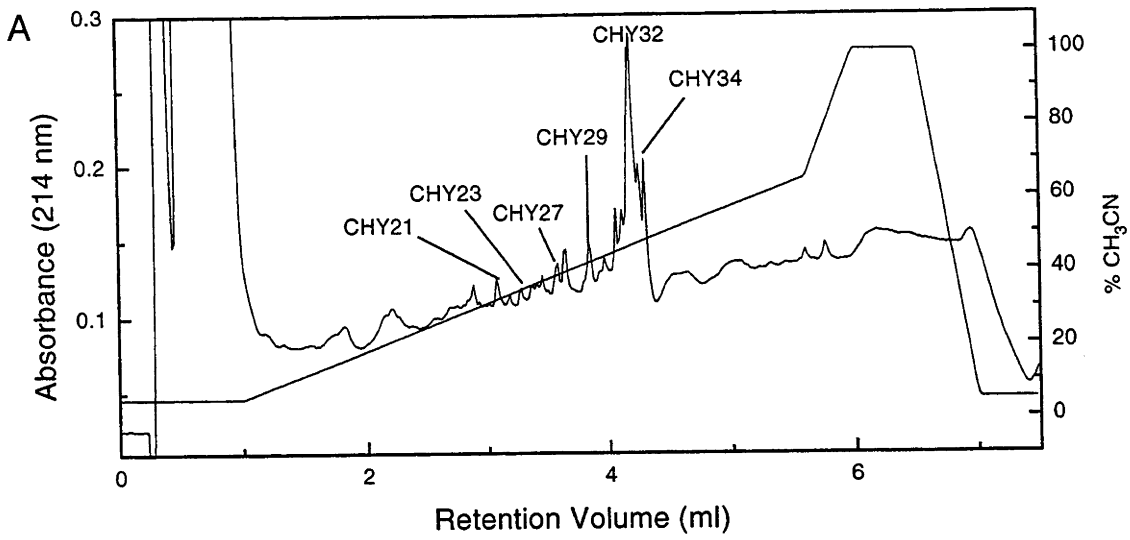
Fig. 6.2. Electrospray mass spectrum of purified *O.anatinus* NGF. The molecular weight of the protein was determined to be 13327.5 (major peak, A)

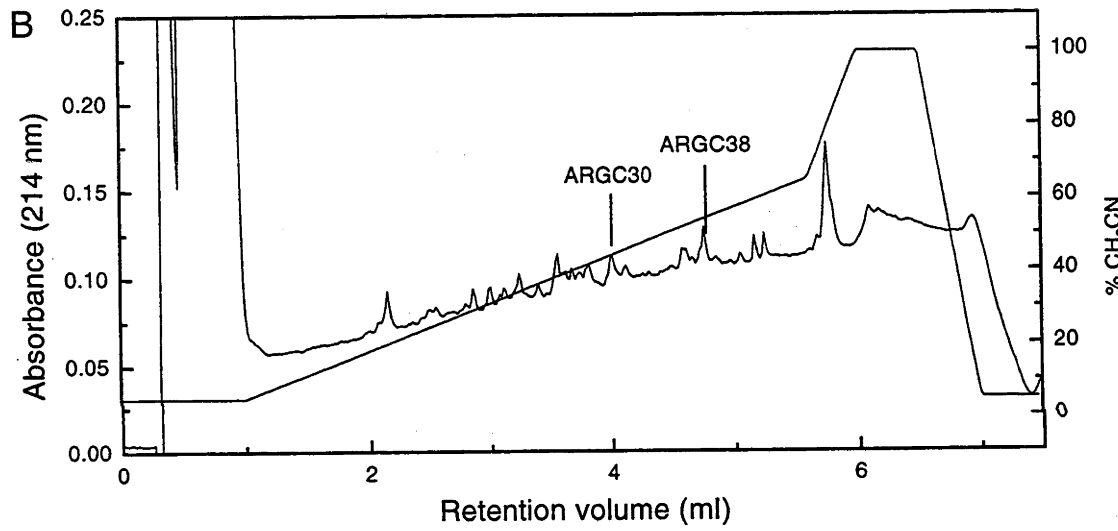
concentrations on neuronal survival. Each curve is based on an experiment performed in duplicate. Under these conditions, the absence of neurotrophic support results in the death of neurones over a 48 hour period. The activity of *Oa*V thus suggested the presence of a neuronal growth factor and NGF was subsequently identified as the factor associated with *Oa*V-elicited inward currents (Chapter 5). The lower potency of *Oa*V compared to purified β-NGF reflects the fact that NGF only constitutes a proportion of total *Oa*V protein. However, the assay may also be affected by cytotoxic or other factors in *Oa*V, as is often the case with NGF-containing snake venom fractions (Kostiza and Meier, 1996).

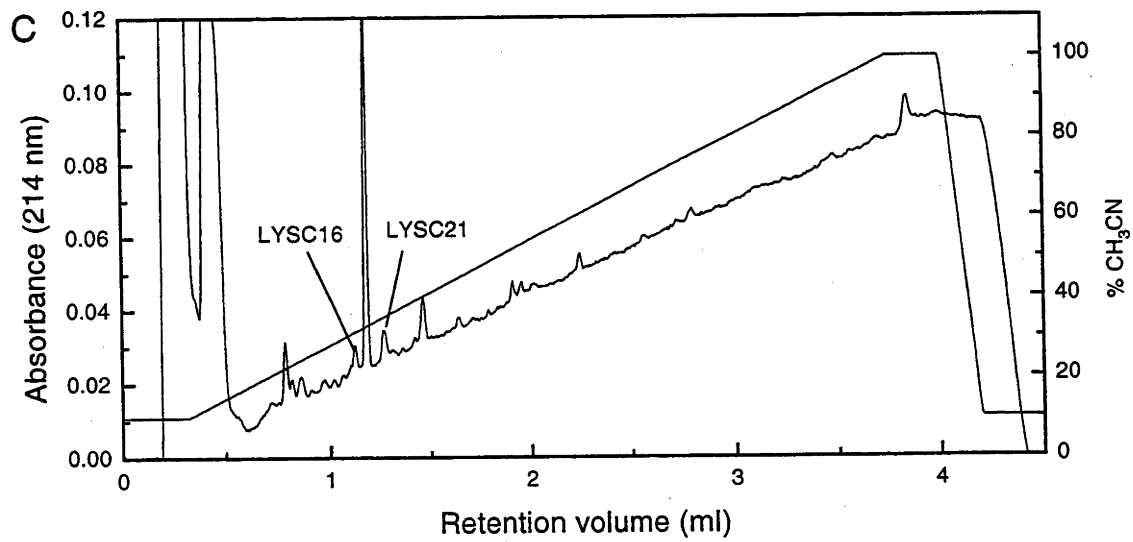
The NGF from *Oa*V was purified to near-homogeneity using a combination of HP-GPC and RP-HPLC, as described in Chapter 5 (see Fig 5.5). It had an apparent relative molecular mass of 12 kDa as determined by SDS-PAGE under non-reducing conditions and analysis by electrospray mass spectrometry revealed a molecular weight of 13,327.5 (Fig. 6.2). This is comparable to Type I snake venom and mouse β-NGFs which have molecular weights of around 13,200 (Fahnestock, 1991; Koyama *et al.*, 1992; Kostiza and Meier, 1996).

*Oa*V NGF was subjected to in-solution proteolytic digestion using chymotrypsin and endoproteinase Arg-C and *in situ* digestion in gel pieces using endoproteinase Lys-C. The resulting fragments were purified by RP-HPLC (μRPC C2/C18, 3.1/10 column for chymotryptic and Arg-C fragments; Brownlee Aquapore OD-300 column for Lys-C fragments) as shown in Fig. 6.3. Peptide fragments were subjected to N-terminal Edman analysis and a large proportion of the protein was mapped from the resulting sequences. The elucidated sequence is shown in Fig. 6.4. Two regions of the protein, corresponding to residues 74-85 and 106 to the C-terminus, could not be determined from peptide fragments and their sequences remain unresolved. Both regions (indicated by dashes in Fig. 6.4 and Xs in Fig. 6.5) are likely to be present given the

FIG 6.3. Reversed phase HPLC purification of proteolytic fragments generated from chymotryptic (A), endoproteinase Arg-C (B) and endoproteinase Lys-C (C) digestion of purified NGF. Lys-C digestion was performed "in-gel" and the fragment purified using an Brownlee Aquapore OD-300 column (Perkin-Elmer) while the other two digests were performed in solution and the fragments purified using a mRPC C2/C18 SC3.1/10 column (Pharmacia). In all cases, eluent A consisted of 0.1% TFA/H_2O and eluent B consisted of 0.1% TFA/CH_3CN. Fragments were labelled according to the proteolytic enzyme and fraction number, and were subjected to N- terminal sequencing, the results of which are shown in Fig. 6.4.


A
Absorbance (214 nm)
0.3
0.2
0.1
CHY32
CHY34
CHY29
CHY23
CHY27
CHY21
100
80
60
40
20
0
% CH3CN
0
2
4
6
Retention Volume (ml)


B
Absorbance (214 nm)
0.25
0.20
0.15
0.10
0.05
0.00
ARGC38
ARGC30
100
80
60
40
20
0
% CH3CN
0
2
4
6
Retention volume (ml)


C
Absorbance (214 nm)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
LYSC16
LYSC21
100
80
60
40
20
0
% CH3CN
0
1
2
3
4
Retention volume (ml)

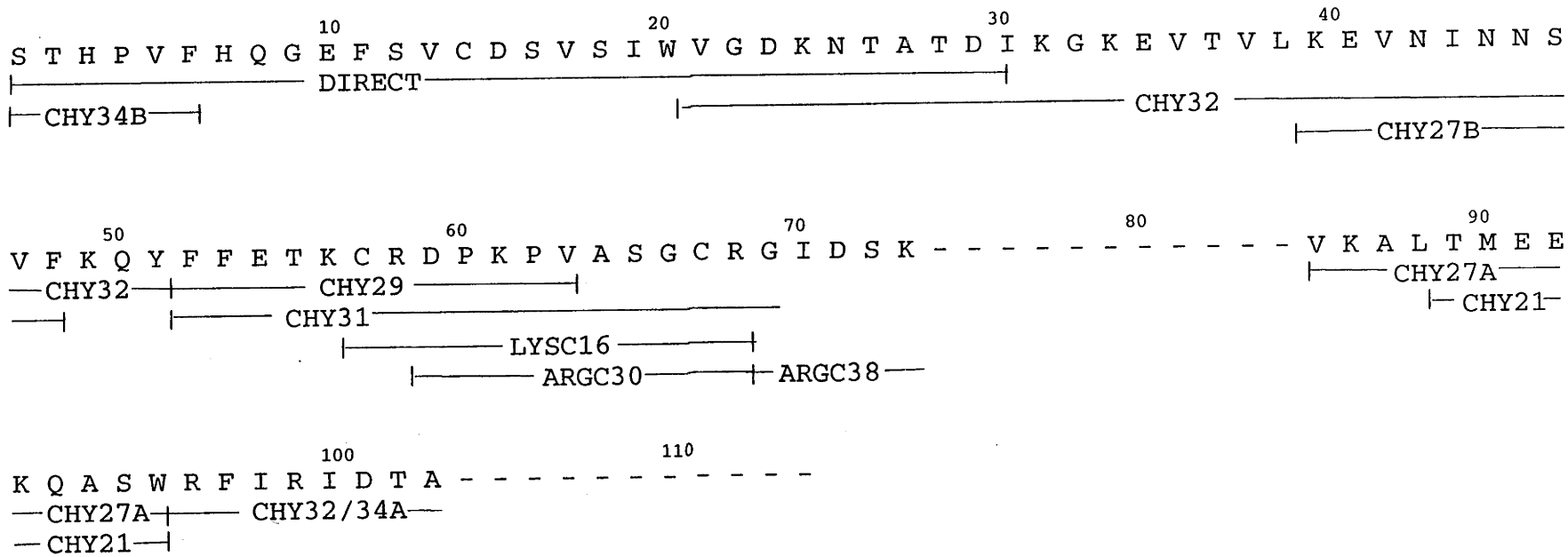


Fig. 6.4. The elucidated partial amino acid sequence of *O. anatinus* NGF. 94 residues of sequence were determined from the N-terminus of intact material as well as from chymotryptic (CHY), endoproteinase Arg-C (ARGC) and endoproteinase Lys-C (LYSC) fragments. Two regions, corresponding to residues 74-85 and 106 to the C-terminus could not be determined from the peptide fragments and remain unresolved.

similarity in mass between *Oa*V NGF and NGFs from other species. However it is possible that, if glycosylated (like Type II NGFs), carbohydrate moieties contribute to the observed mass, *i.e.* a reduced mass resulting from "missing" sequence elements may be compensated for by carbohydrate.

A comparison of NGF sequences from *Oa*V and from other species, including from the venom of Viperid (*Vipera russelli*) and Elapid (*Naja naja atra*) snakes, is given in Fig. 6.5. In the 94 residues of sequence elucidated, *Oa*V NGF exhibits 90% identity with mouse NGF and 67% identity with *Naja naja atra* venom NGF. One highly conserved site, corresponding to residue 25, is substituted in *OaV* NGF. Residue 61, which is conserved except in chicken NGF, is also substituted in *Oa*V NGF. Residues 56-97 of *O. anatinus* NGF have been previously elucidated from genomic DNA sequencing (Kullander *et al.*, 1997) and are identical to the sequence obtained here for the *Oa*V NGF (Fig. 6.5).

Discussion

*Oa*V produces a dose-dependent, NGF-like increase in the survival of sensory ganglion neurones *in vitro*. Subsequently, it has been shown to contain an NGF-like protein, which has been purified and most of its amino acid sequence determined. It was found to share a greater degree of homology with mammalian NGFs than with snake venom NGFs.

Structure

By electrospray mass spectrometry, *Oa*V NGF was found to have a molecular weight of 13,327.5, indicating its similarity to mouse β-NGF and Type I snake venom NGFs. No evidence of glycosylation (*i.e.* like type II snake venom NGFs) was apparent from the Edman sequencing results, although further analyses may prove otherwise. *Vipera russelli russelli* NGF (a Type II NGF) is N-glycosylated at Asn^{21} within the signal

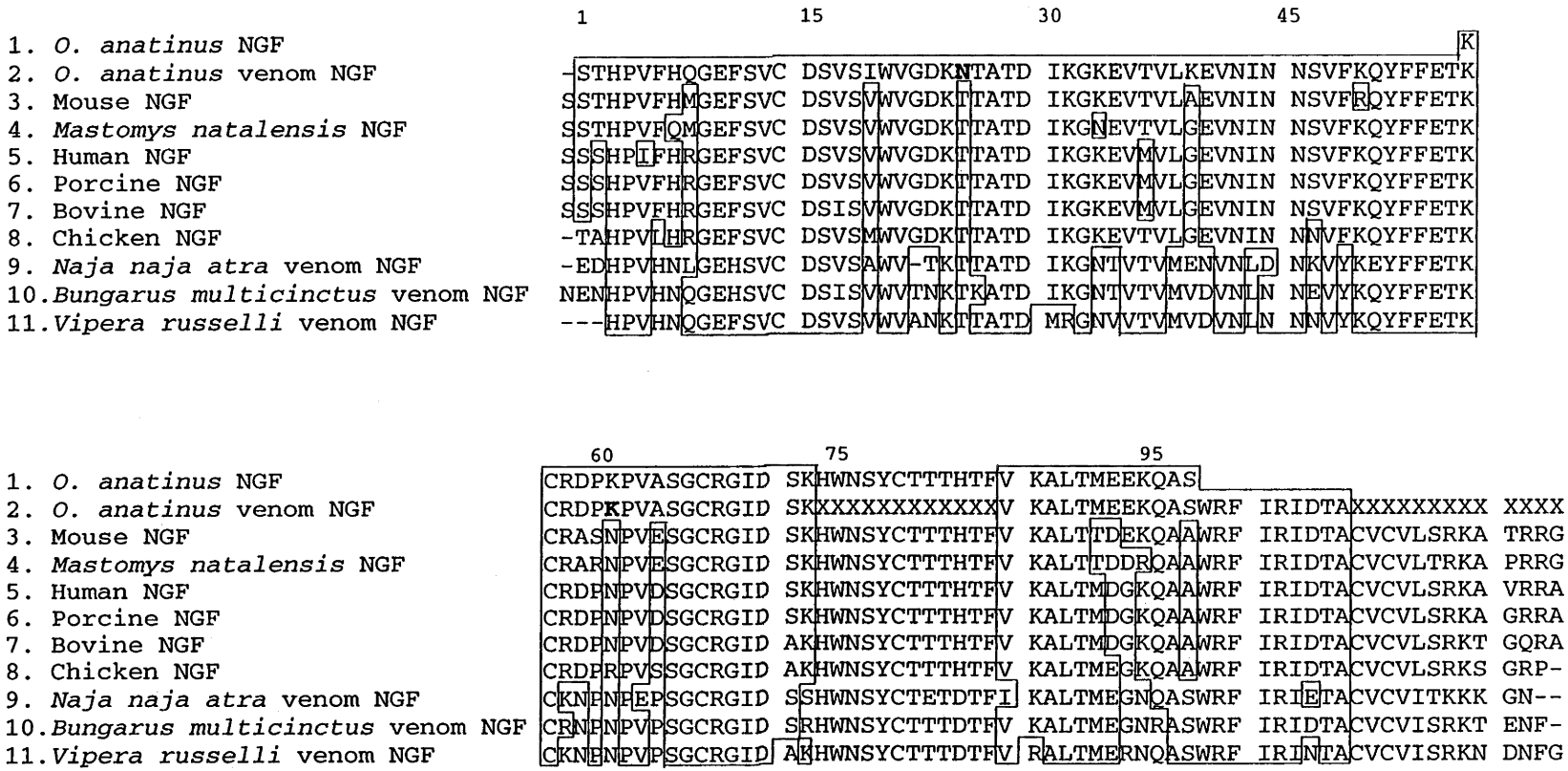


Fig. 6.5. Comparison of the amino acid sequence of *O. anatinus* venom NGF with NGFs from other species. Note that *O.anatinus* venom NGF shares a greater degree of homology with mammalian than snake venom NGFs. One otherwise conserved site, corresponding to residue 25 and one highly conserved (except in chick NGF) site, corresponding to residue 61, are substituted in *O.anatinus* NGF (indicated in boldface). Common names of indicated species are: *Mastomys natalensis*, African Soft-furred rat; *Naja naja atra,* Taiwan cobra; *Bungarus multicinctus,* Chinese krait; *Vipera russelli,* Russell's viper.

sequence, Asn-X-Thr(Ser) (Koyama *et al.*, 1992), which is not present in *Oa*V NGF (*V.r.russelli* NGF Asn[21] corresponds to *Oa*V NGF Asp-23).

*Oa*V NGF migrates as an approximately 12 kDa protein on SDS-PAGE under non-reducing conditions. It thus does not appear to be a disulphide-linked dimer and therefore differs from Type III NGFs such as those from *Bungarus multicinctus* and *Bitis arientans* venoms.

Unlike *Crotalus adamanteus* venom from which a γβ-like subunit complex was recovered (Perez-Polo *et al.*, 1978), no other NGF subunits have been recovered from *Oa*V. This suggests *Oa*V NGF is not a Type IV NGF (Kostiza and Meier, 1996).

Pro-inflammatory and algogenic effects of NGF

Aside from its role during development, it has been clearly established that NGF affects non-neuronal cells, especially those derived from haematopoetic stem cells (Kostiza and Meier, 1996). NGF potently elicits histamine release[17] from mast cells (Pearce and Thomson, 1986) and this activity appears to be mediated specifically through high affinity *TrkA* receptors (Horigome *et al.*, 1993). Histamine increases vascular permeability and thus may facilitate the diffusion of venom components. NGF also increased the number of mast cells at the site of injection (Aloe and Levi-Montalcini, 1977) and administration of anti-NGF antibodies decreased the number of free and tissue-adherent mast cells in rodents (Aloe, 1988), suggesting a possible role in mast cell hyperplasia. NGF induces the differentiation of neutrophils, macrophages, eosinophils and basophils from progenitor cells (Matsuda *et al.*, 1988a and b), has a chemotactic effect on polymorphonuclear leukocytes (Gee *et al.*, 1983) and promotes basophil degranulation (Kostiza and Meier, 1996). These cytokine-like effects may

[17] However, only components from a 10 kDa ultrafiltrate were assayed for histamine-releasing activity in this study (see Chapter 4) and the histamine-releasing activity of *Oa*V NGF was not investigated

contribute to the pro-inflammatory effects of *Oa*V.

The potential therapeutic efficacy of NGF in preventing the onset of symptoms of neurodegenerative diseases has prompted experimental studies on the effects of systemic NGF administration and there is a remarkable similarity between the observed effects of NGF and *Oa*V. For example, the subcutaneous or intravenous injection of recombinant NGF into healthy human subjects resulted in pain and hyperalgesia which lasted up to 7 weeks (Petty *et al.*, 1994; Dyck *et al.*, 1997). Mechanical and heat hyperalgesia have also been described in experimental animals following NGF administration (Lewin *et al.*, 1993) and similarities between NGF-induced hyperalgesia and that associated with tissue injury have led to the suggestion that expression of NGF in inflamed tissue may be a critical link between tissue injury and hyperalgesia (Lewin and Mendell, 1993).

The mechanism of NGF-induced hyperalgesia is complex and appears to involve a sympathetic component, mast cell degranulation and an effect on sensory neurones (Woolf *et al.*, 1996). Sympathectomy abolishes the mechanical hyperalgesia and markedly reduces the thermal hyperalgesia induced by NGF in rats (Andreev *et al.*, 1995; Woolf *et al.*, 1996), suggesting that NGF-induced hyperalgesia requires the activation of sympathetic post-ganglionic neurones. However, depletion of mast cell granules and the administration of 5-HT receptor antagonists abolishes early (< 3 h) NGF-induced heat hyperalgesia (Lewin *et al.*, 1994), suggesting that mast cell degranulation also plays a role in the acute phase at least. In addition, the NMDA receptor antagonist, MK-801 blocks the late phase (> 7h) thermal hyperalgesia produced by NGF, suggesting a central mechanism (Lewin *et al.*, 1994) which may follow the retrograde transport of NGF in sensory neurones (Woolf *et al.*, 1996). Subsequent upregulation of both neuropeptide (Lindsay and Harmer, 1989; Donnerer *et al.*, 1992; Leslie *et al.*, 1995) and Na^+ channel expression (Toledo-Aral *et al.*, 1995)

and, perhaps, hyperinnervation of peripheral tissue (Albers *et al.*, 1994; Leslie *et al.*, 1995) may also contribute to the NGF-induced hyperalgesia.

β-NGF, like *Oa*V (Kellaway and LeMessurier, 1935), also produces a dose-dependent hypotensive effect in rats following intravascular administration (Yan *et al.*, 1991). Prior depletion of histamine from mast cells using compound 48/80 and treatment with the antihistamine, chlorpheniramine, rendered the animals refractory to NGF, suggesting the effect is mediated primarily through histamine release from mast cells (Yan *et al.*, 1991). Thus, in addition to ovCNP-39, NGF may contribute to the hypotensive effects of *Oa*V.

What is the function of NGF in O. anatinus venom?

The role of NGF in *Oa*V may be related to its role in snake venom and mouse submaxillary glands. Several, largely speculative, theories have been proposed. It has been suggested that both venom and submaxillary glands may function simply to remove excess NGF from the circulation (Hogue-Angeletti and Bradshaw, 1979). It is also possible that the glands secrete NGF, like other sympathetic target organs, to regulate sympathetic innervation during development (Hogue-Angeletti and Bradshaw, 1979). However, this raises the question as to why other organs receiving extensive sympathetic input do not produce quantities of NGF anywhere near as large as those found in venom and submaxillary glands.

Interestingly, the NGF content of murine submaxillary glands is sexually dimorphic. NGF is found in far greater abundance in the submaxillary gland of the male mouse compared to the female (Thoenen and Barde, 1980). A parallel situation exists in *O. anatinus*, where the NGF-secreting crural gland is found only in the male. In the mouse, testosterone modulates NGF synthesis (Thoenen and Barde, 1980) and it may also be a key regulator of crural gland secretory activity, given that phasic

hypertrophy of the crural and secondary sex glands coincide (Temple-Smith, 1973). Intriguingly, intraspecific fighting between male mice following social isolation produces a massive release of NGF from the salivary glands into the bloodstream (Aloe *et al.*, 1986). The observed adrenal hypertrophy following NGF administration suggests that the adrenal gland is a primary target for this systemic NGF release (Aloe *et al.*, 1986). The crural venom apparatus is believed to play a key role in the resolution of similar aggressive behavioural encounters between male *O. anatinus* (see Chapter 1) and it is conceivable that an associated release of NGF into the bloodstream also occurs. Perhaps associated adrenal gland hypertrophy following an aggressive encounter would have the advantage of increasing adrenaline-mediated "fight-or-flight" reactions[18] during subsequent encounters. Unfortunately, a sex difference in snake venom NGF concentrations has not been investigated (Kostiza and Meier, 1996) making any speculation on a similar function in snakes difficult.

NGF may potentiate or contribute to the toxic effects of venom and saliva. Crude mouse submaxillary gland extracts are lethal when injected intraperitoneally into adult mice at relatively low protein concentrations (Liuzzi and Angeletti, 1968). Extracts from male mouse submaxillary glands are much more potent in this regard than those from female mice (Liuzzi and Angeletti, 1968), suggesting that NGF is responsible for, or contributes to, the lethal effects. Submaxillary gland extracts from the venomous shrews (*Blarina brevicauda*, *Solenodon paradoxus* and *Neomys sp.*) produce dyspnoea, hypotension, convulsions and death following administration to experimental animals (Dufton, 1992). In humans, shrew bites result in oedema, a burning sensation and "shooting" pains which may persist for days (Dufton, 1992). The parallels between these effects and those of *Oa*V and NGF, in addition to the presence of NGF in other rodent submaxillary glands (NGF is also abundant in the

[18] such reactions include a decrease in visceral organ activity and stimulation of heart and skeletal muscles.

submaxillary gland of the African rat, Mastomys natalensis, Fahnestock, 1991), raise the possibility that NGF is also a component of venomous shrew saliva.

NGF may also contribute to pain and hyperalgesia associated with both *Oa*V and snake envenomation. Kostiza and Meier (1996) have estimated that 50 μg of NGF could be injected from an elapid snake bite. For a 75 kg human, this equates to around 0.7 μg kg^{-1}. Such a dose would be more than sufficient to produce long-lasting (> 12 days) hyperalgesia in humans following subcutaneous injection, which has been described following NGF doses as low as 0.03 μg kg^{-1} (Petty *et al.*, 1994). However, reports of lasting hyperalgesia following snake envenomation are not apparent in the literature, although at least one patient receiving a *Naja naja* bite was believed to have presented with prolonged (several months) hyperalgesia (John Klein, http:\\www.cobra.org, pers. comm.). Clinical studies of snake bite symptoms and management are necessarily dominated by the early resolution of life-threatening effects, *e.g.* neurotoxicity and coagulopathy. Where prolonged pain has been reported, it is commonly associated with myonecrotic and haemorrhagic effects (*e.g.* Clement and Pietrusko, 1978). Another complicating factor is the administration of antivenom which may contain anti-NGF antibodies, thus diminishing any NGF-induced hyperalgesia in envenomed patients. Notwithstanding these considerations, reports of severe pain following envenomation by snakes whose venom contain NGF, are common. For example, venom from members of the *Bothrops* and *Crotalidae* genera, as well as from *Bungarus caeruleus* and *Naja naja naja,* all contain NGF and produce marked pain (Ribeiro and Jorge, 1997; Teixeira *et al.*, 1994; Plowman *et al.*, 1995; Guisto, 1995; Theakston *et al.*, 1990).

Conclusion

The presence of NGF in *Oa*V is of great interest, not only because it is present in snake venoms but also because many parallels exist between the activities of *Oa*V and NGF from other mammals. In particular, the ability of NGF to produce pain and long-lasting hyperalgesia is an exceptional feature which may underlie similar effects observed following *O. anatinus* envenomation. *Oa*V NGF also appears to contribute to the inward current activity of *Oa*V in DRG neurones and this activity may contribute to the algogenic effects of *Oa*V. *Oa*V NGF is structurally very similar to both mouse and Type I snake venom NGFs, suggesting a similarity in biological activity. The much higher degree of sequence homology shared between *Oa*V and mouse NGFs compared to snake venom NGFs, is consistent with the other mammalian characteristics of *O. anatinus*.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE DIRECTIONS

General discussion

Pain and oedema are the most consistent features of *O. anatinus* envenomation and the major objective of this study was to identify and characterise components and mechanisms likely to contribute to these effects. Initially, the major components of *O. anatinus* venom (*Oa*V) were characterised at the protein sequence level to ascertain any homology with known polypeptides which have pro-inflammatory effects. Two components, ovCNP-39 and NGF, were subsequently characterised in detail, and the possibility that *Oa*V may directly activate nociceptive neurones was investigated.

Overall, 19 separate components have been detected in *Oa*V by a combination of SDS-PAGE and reversed phase HPLC. Several of these exhibited a difference in their level of relative expression depending on the season in which the venom was collected, while the remainder showed no apparent trend. This supports prior evidence of seasonal variation in venom composition and is consistent with seasonal changes in crural gland histology (Temple-Smith, 1973). Although some *Oa*V components were demonstrated to be related to specific protein/polypeptide families, no intuitively obvious functions were suggested by their amino acid sequences except in the case of the C-type natriuretic peptide (ovCNP-39) and p12/nerve growth factor (NGF).

The peptide, ovCNP-39, which constitutes a relatively high mole fraction of venom components, was found to release histamine from mast cells and to produce oedema. ovCNP-39 had a comparable potency to eutherian CNP with regard to cGMP elevation in cultured vascular smooth muscle cells. Thus, ovCNP-39 may contribute to

the vasodilatory and hypotensive effects of *Oa*V both through the release of histamine, which is a potent vasodilator and through cGMP elevation in vascular smooth muscle.

The longevity and severity of the hyperalgesic effects of *Oa*V are, however, not reconcilable with the acute activity of mediators such as histamine (Keele and Armstrong, 1964). Hence, the possibility of a direct effect of *Oa*V on sensory neurones was investigated using sensory ganglion neuronal cell bodies *in vitro*, which express properties and chemical sensitivities similar to polymodal nociceptors *in vivo* (Gold *et al.*, 1996a). *Oa*V was found to produce a lasting inward current in these neurones and this appears to be the first example of a direct electrophysiological effect of a venom on nociceptive neurones. Following envenomation, nociceptive endings would almost certainly be exposed to higher concentrations of *Oa*V for a longer time (*i.e.* greater than 10 sec) and the conditions used to elicit the inward currents in this study are therefore probably conservative in comparison to what might be expected under normal circumstances. It is thus likely that a direct effect on nociceptive neurones contributes substantially to the painful effects of envenomation.

The inward current activity was associated with *Oa*V NGF which shares 90 % identity with mouse β-NGF in the 94 residues of partial sequence obtained in this study. However mouse β-NGF does not activate an inward current in the same preparation which suggests a possible synergy between *Oa*V NGF and another component in the venom, although no consistently co-purified component was detected by SDS-PAGE.

Interestingly, the biological activities of NGF and *Oa*V are remarkably similar. A single subcutaneous dose of recombinant human NGF in human subjects produces localised hyperalgesia persisting for up to 7 weeks (Petty *et al.*, 1994), a time-course similar to that which follows *O. anatinus* envenomation (Fenner *et al.*, 1992). NGF

also produces hypotension (Yan *et al.*, 1991) and may, in addition to ovCNP-39, contribute to the hypotensive effect of *Oa*V in experimental animals.

Like *Oa*V, venoms from several snake genera also contain natriuretic peptides, NGF and Kunitz-type protease inhibitors (some of which have ion channel blocking activities). In these respects, *Oa*V may be considered to resemble snake venoms. However, it may also be regarded as akin to mammalian exocrine secretions, such as the mouse submaxillary gland secretion, given that *Oa*V NGF shares 90 % homology with mouse submaxillary β-NGF and only around 67 % with snake venom NGFs. Curiously, no case reports of lasting hyperalgesia following snake envenomation are apparent in the literature, despite the known hyperalgesic effects of NGF. This may be related to structural differences between snake venom and human NGFs, such that snake venom NGF is recognised as foreign and destroyed by the human immune system. On the other hand, *Oa*V NGF is structurally much more similar to mammalian NGFs and may therefore escape immunological targeting. Presumably, the absence of immunological reactions to *Oa*V components following intraspecific envenomation would also be important for maintaining its efficacy in *O. anatinus* and preventing the development of resistance to subsequent envenomations. However, if an immunological reaction did occur following intraspecific envenomation, it may have the advantage of promoting autoimmune-mediated crural gland regression in the victim.

This study has described several ways in which *Oa*V may produce noxious, pro-inflammatory effects and therefore lends support to its putative involvement in intraspecific conflicts between reproductively active male *O. anatinus*. *Oa*V may thus facilitate the maintenance of home range boundaries and allow dominant males to monopolise breeding females. This role alone appears to set *Oa*V apart from snake and other animal venoms wherein the primary role lies in food procurement and self-

defence (not intraspecific) and further suggests its similarity to mammalian exocrine gland secretions. Notable examples include the mouse submaxillary gland, which releases large amounts of NGF during intraspecific fighting between male mice and, shrew submaxillary glands, which produce a toxic secretion of unknown function but with similar properties to *Oa*V.

Suggestions for future experiments

The design of experiments aimed at elucidating the factors and mechanisms which underlie the pain-producing and hyperalgesic effects of *Oa*V, presents a number of difficulties. Pain and hyperalgesia are subjective sensations and this makes them difficult to measure experimentally. In addition, pain is a complex phenomenon and many factors may contribute to its induction and perceived severity. For example, the mechanisms underlying NGF-induced hyperalgesia appear to involve both sympathetic and sensory neurones as well as non-neuronal inflammatory cells (Lewin *et al.*, 1994; Woolf *et al.*, 1996) and therefore the mechanisms of *Oa*V-induced hyperalgesia may be equally complex, if for no other reason than the fact that *Oa*V contains NGF.

As discussed previously, these effects may be characterised by measuring the limb withdrawal latencies of experimental animals as an index of pain and hyperalgesia, and various inhibitors and antagonists could be administered systemically to probe postulated mechanisms. However, apart from potential ethical implications, the amount of detailed mechanistic information which may be obtained through the use of these techniques is limited. Another approach would be to assay for *Oa*V and *Oa*V fraction-induced release of inflammatory mediators *in vivo* and *in vitro*. Apart from mast cell histamine release, this approach was not explored in the present study but may provide useful information on the role of mediators, which produce prolonged algetic responses, in the induction of *Oa*V mediated pain and hyperalgesia. Several

approaches could be taken. Firstly, systemic (assayed from serum) and local (assayed from inflammatory exudate) increases in the levels of cytokines, such as IL-6 and TNF-α, and other factors believed to play a role in nociceptor activation, may be assayed following the administration of *Oa*V to experimental animals. This could be accomplished using commercially available quantitative enzyme linked immunosorbent assays (ELISA) (Sofer *et al.*, 1996), or by measuring the ability of antisera raised against these factors to attenuate *Oa*V-induced hyperalgesia. Alternatively, *in vivo* inflammatory cell infiltration into subdermal tissue following *Oa*V administration could be monitored and may suggest inflammatory pathways (see Fig. 1.1) likely to be involved in *Oa*V hyperalgesia.

KLP

The Kunitz-type protease inhibitor-like protein (KLP) from *Oa*V shares some homology with certain snake venom neurotoxins, such as the K^+ channel blocker dendrotoxin (Harvey, 1997). It is therefore possible that KLP may have a similar ion-channel-blocking activity. Such an effect may be determined by isolating whole cell K^+ currents in DRG neurones by, for example, substituting choline-Cl for NaCl and $CoCl_2$ for $CaCl_2$, and determining whether KLP is capable of reducing the amplitude of remaining currents. These may be further dissected using apamin, which blocks $I_{K(Ca)}$ and, dendrotoxin, which blocks I_{KA} (Hille, 1992). An alternative approach would be to screen expression systems, such as oocytes or HEK cells, which express particular K^+ channel subtypes, as Kunitz-like neurotoxins may target specific subtypes not be present or detectable in DRG neurones.

The elucidation of both N-terminal and internal segments of amino acid sequence from *Oa*V proteins, such as KLP, will facilitate the future cloning and expression of *Oa*V proteins and this may confirm the suspected homologies outlined here and

provide viable quantities of individual components for biological activity screening experiments such as those described above.

ovCNP-39

The difference between the ovCNP-39 isoforms, ovCNP-39A and B, was not resolved in this study. The heterogeneity is unlikely to be conformational given that their sequences and molecular masses are identical. The difference is localised to the N-terminal 11 residues and may result from an unusually stable peptidyl-prolyl *cis-trans* isomerisation. Preliminary circular dichroism spectroscopy did not reveal any difference and a more highly resolving technique, such as two-dimensional NMR will probably be required to resolve the *ex vivo* ovCNP-39 heterogeneity. However, the large (mM) peptide concentrations required limits the feasibility of using NMR until larger quantities of material become available.

OaV-activated inward currents

The precise mechanism by which *Oa*V activates inward currents in sensory neurones also remains to be elucidated. The delay to the onset of the response, in addition to its apparent dependence on Ca^{2+} release from intracellular stores, suggests the involvement of a metabotropic pathway. Furthermore, NGF binding to TrkA has been shown to phosphorylate phospholipase C-γ (Vetter *et al.*, 1991), leading to accumulation of IP_3 and release of Ca^{2+} from intracellular stores (De Bernardi *et al.*, 1996). Whether or not the *Oa*V current is dependent on this process could be investigated by establishing whether an antibody specific to phospholipase C-γ can modulate the response (*e.g.* see Yassin and Abrams, 1991). Alternatively, the *Oa*V-induced current may involve G-protein activation. This may be established in several ways, the most obvious of which would be to determine the effects of pertussis toxin or the non-hydrolysable GTP analogue GTP-γ-S on the activity. Removal of GTP from

the patch pipette would also be expected to abolish the response if it involved G-protein activation.

The evidence for the dependence of the inward current on Ca^{2+} release from intracellular stores is based solely on the ability of thapsigargin to inhibit the current at this point. This could be confirmed using a different Ca^{2+}-ATPase inhibitor, such as cyclopiazionic acid. Alternatively, a ratiometric, calcium-sensitive fluorophore- (*e.g.* indo-1) based single-cell microfluorimetry or alternative fluorescence technique may be used to quantify elevations in Ca^{2+} concentration and could be used in combination with thapsigargin, cyclopiazonic acid or low extracellular Ca^{2+}, to identify the source of Ca^{2+} contributing to its intracellular elevation. The type of Ca^{2+} store involved may be determined by investigating the relative effects of Ca^{2+} release channel inhibitors on the *Oa*V-activated inward current, such as heparin, which is an IP_3 channel inhibitor, or ryanodine, which inhibits CICR.

p12/NGF was not purified to homogeneity and p12/NGF-containing fractions from ion exchange or chromatofocusing fractions appeared not to be as potent as those obtained from HP-GPC. This raised the possibility of a synergy between p12/NGF and another *Oa*V component in the HP-GPC active fraction which may elute differently during ion-exchange HPLC or chromatofocusing. This possibility may be tested by establishing whether inactive *Oa*V fractions, not containing NGF, are able to generate inward current activity when combined with mouse β-NGF. An alternative would be to remove *Oa*V NGF from active fractions, using immunoprecipitation or immunoaffinity chromatography, and attempt to rescue the resulting loss of activity by adding mouse β-NGF.

Concluding comments

Primary amino acid sequences of *O. anatinus* venom polypeptides were obtained for the first time in this work and the data obtained will be indispensable for the future synthesis of *Oa*V peptides and the development of expression systems for larger proteins. A novel-length C-type natriuretic peptide was identified in *Oa*V and this work has demonstrated its likely contribution to the oedemagenic and hypotensive effects of *Oa*V. The possibility of a direct effect of a venom on nociceptive neurones was investigated. In small-medium diameter dorsal root ganglion neurones, many of which are putative nociceptors, a brief application of *Oa*V produces large Ca^{2+}-dependent inward currents lasting, on average, for several minutes. Such an effect at nociceptive terminals may well be responsible for the intractable, excruciating pain which accompanies *O. anatinus* envenomation. This is the first demonstration that a venom may act directly on nociceptive neurones and raises the possibility that it occurs following severely painful envenomation by other species. The activity appeared to be associated with NGF which, interestingly, is found in many snake venoms and, like *Oa*V, produces long-lasting pain and hyperalgesia. Most of its amino acid sequence has been elucidated and its close relatedness to other mammalian NGFs has important functional and evolutionary implications.

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