High affinity binding of neuropeptide Y to a polypeptide component from the venom of *Conus anemone*

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CONTEXT AND IMPLICATIONS OF THE PRESENT STUDY.

Plant and animal (e.g. snake, scorpion) toxins have proven to be extremely useful in defining key components of vital physiological systems. As extensively reviewed in Trends in Neurosciences (supplement on neurotoxins, June 1996) neuromuscular and neuronal transmission may be blocked at the level of ion channels, specific receptors, G-proteins and enzymes. Interest in the action mechanism and potential therapeutic use of *Conus* toxins is only recent, but it is rapidly growing. The family of Conidae consists of more than 300 members of marine gastropods. They possess potent venoms which are used primarily in the capture of prey organisms such as worms, other molluscs and fish. Conotoxins have, so far, been shown to interact with ion channels and certain receptors. To our surprise, we found that the venom of *Conus anemone* contains a peptide component that is able to bind the neurotransmitter Neuropeptide Y (NPY). NPY controls a wide variety of functions including psychomotor activities, cognitive functions, sexual behaviour, food intake and blood pressure regulation. The characterization and purification of the NPY-binding peptide component of *Conus anemone* venom, termed ANPY-toxin (from *Conus anemone* -NPY), has been presented in the Ph. D. thesis of Dr. Eva Czerwiec (June 11, 1996) and will be presented in two international publications (Czerwiec et al., 1996a, 1996b). The contents of the two publications have been combined to form the present report.

The present data constitute the first evidence that *Conus* venoms contain components which are capable of interacting with peptide neurotransmitters. This may only constitute the "tip of the iceberg" since, so far, no attention has been paid to direct toxin-neurotransmitter interactions. This concept merits to be explored further as it could give rise to a whole new class of toxins from animal, plant and bacterial origin. Such toxins could be used as "antagonists" in pharmacological and physiological studies, for determining the distribution of neurotransmitter in tissue slices, for the purification of peptide neurotransmitters. The major advantage of such toxins over existing antibodies is that they may be comparatively small and constitute of a single peptide chain. These properties would also greatly facilitate the artificial production of such toxins by cultured cell lines.

INTRODUCTION

The family of Conidae consists of more than 300 members of marine gastropods. They possess potent venoms which are used primarily in the capture of prey organisms such as worms, other molluscs and fish (Endean and Rudkin, 1965). All *Conus* species possess a similar venom apparatus (Olivera et al., 1988) which is composed of a muscular venom bulb (acting as a pump), a long hollow duct (where the venom is made), the pharynx containing disposable, harpoon-like teeth and the proboscis (a tube forming part of the mouth) (Fig. 1). Observations from aquaria reveal that when a cone is ready to attack its prey, it transfers one of the harpoon-like teeth into the proboscis. The prey is speared with the tooth and, at the same time, the muscular bulb contracts and the venom is pushed trough the duct, the pharynx, the proboscis and finally through the hollow tooth into the victim. This provokes instant paralysis, and the snail may now engulf its prey with its distensible stomach. Cones are also known to use their venom apparatus to defend themselves. In this respect, piscivorous species such as *Conus geographus* and molluscivorus species such as *Conus textile* have provoked human deaths (Endean and Rudkin, 1965 Rice and Halstead, 1967). Severe injuries have also been caused by stings of other *Conus* species, even vermivorous ones.
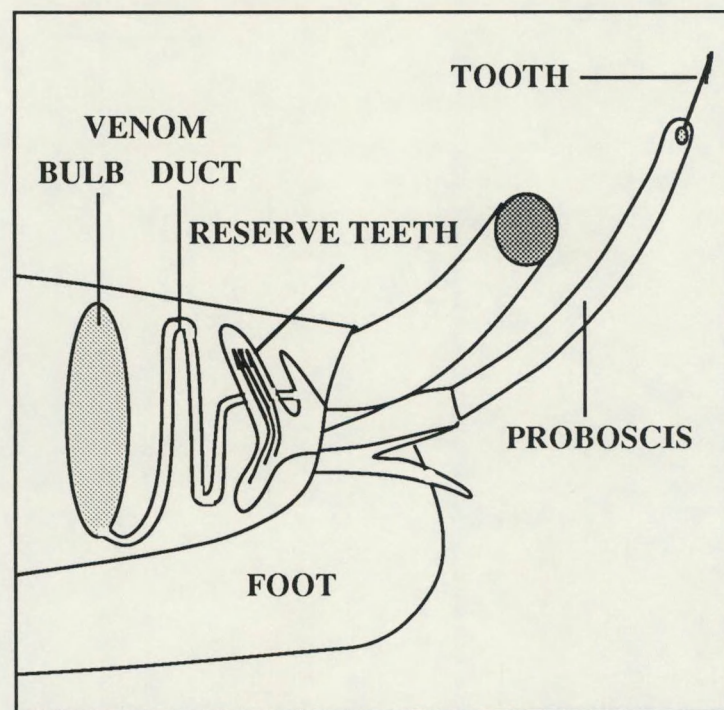

Figure 1: Schematic representation of the *Conus* venom apparatus

Plant and animal (e.g. snake, scorpion) toxins have proven to be extremely useful in defining key components of vital physiological systems. Interest in the action mechanism and potential therapeutic use of *Conus* toxins is only recent, but it is rapidly growing. Whereas, in the early sixties, Endean and coworkers were still investigating the lethal doses of isolated crude *Conus* venoms on different animal species (Endean and Rudkin, 1965), subsequent pharmacological

and biochemical research by Kobayashi and coworkers in Japan (Kobayashi et al., 1982) and by Olivera and coworkers in the USA (Olivera et al., 1985, 1988, 1990, 1991, 1994) led to the concept that *Conus* venoms are complex mixtures of peptides and polypeptides that interact with a variety of physiological targets. Interestingly, it was also found that the nature and occurrence of these toxins is species-dependent.

The majority of the investigated conotoxins block neuromuscular and neuronal transmission by interacting with pre- and postsynaptically located ion channels or ligand-gated ion channels (Gray et al., 1988; Olivera et al., 1985; Olivera et al., 1994). They are often small peptides, generally 10-30 amino acids long with a high cysteine content and they have been classified according to their physiological activity as well as according to their structure (Gray et al., 1988; Olivera et al., 1985, 1990, 1991). The μ-conotoxins, μO-conotoxins and δ-conotoxins bind to voltage operated Na^+ channels and the ω-conotoxins interact with presynaptically located voltage sensitive Ca^{2+} channels. The α- and αA-conotoxins are blockers of the nicotinic acetylcholine receptor, and the conantokins modulate the NMDA-glutamate receptor. Several of these small conotoxins have a high target specifity which makes them useful as selective pharmacological tools (Cruz et al., 1985; Gray et al., 1988; Groebe et al., 1995) and therapeutic agents (Xiao et al., 1995). Some larger conotoxins have been isolated as well, and were shown to interact with voltage operated Na^+ channels (striatoxin (25 kDa) from *Conus striatus*) and to control the Ca^{2+} homeostasis (eburnetoxin (28 kDa) from *Conus eburneus*; tessulatoxin (55 kDa) from *Conus tessulatus* and two polypeptide toxins from *Conus distans* (24 kDa and 25.5 kDa) (Kobayashi et al., 1982a, 1982b, 1983; Partoens et al., 1996; Schweitz et al., 1986).

However, many hormone- and neurotransmitter receptors generate signals in the cells by stimulating G-proteins which, in turn, regulate enzymatic functions (e.g. adenylate cyclase) at the cytoplasmic side of the cell membrane. We have investigated these receptors for nearly two decades and we are especially interested in their intimate structure and their pharmacological characteristics.In this context, it has now become possible to investigate them directly by binding of radioactively labelled hormone- or neurotransmitter analogs: i.e. the radioligands. Such experiments can be performed on cell membranes which are isolated from various tissues and organs. With this approach, animal material can be obtained from a local slaughter house, thereby avoiding the unnecessary killing of laboratory animals. Since these receptors do not necessitate the opening of ion channels to generate signals, very little attention had been paid to their ability to interact with conotoxins. Recently, certain conotoxins have indeed been shown to interact with hormone or neurotransmitter receptors that are not constituents of ion-channels. Conopressins, for example, are vasopressin/oxytocin analogs that are agonists of the vasopressin receptor (Cruz et al., 1987; Fox et al., 1987). Initial screening studies, performed in our laboratory, indicated that the venoms of certain conidae contain peptide components

which are capable of interacting with receptors for e.g. adrenaline, dopamine, serotonin and acetylcholine. These peptide components have a high molecular weight (>10 kDa) and, as a typical example, it was shown that the venom of *Conus tessulatus* is able to discriminate between the M1- and M2-muscarinic receptors as well as between 5-HT$_{1A}$ receptors and α_2-adrenergic receptors (Czerwiec et al., 1989, 1993; De Vos et al., 1991).These findings indicate that conotoxins are particularly succesful discriminatory tools for the division of hormone and neurotransmitter receptors into different subtypes and, in certain instances, their discriminatory power may well exceed that of other natural or synthetic ligands. This is illustrated by the case of muscarinic receptors for which synthetic ligands such as pirenzepine provide a much poorer distinction between the M1- and M2-subtypes than the venom of *Conus tessulatus* (Czerwiec et al., 1993).

Receptors for neuropeptides such as neuropeptide Y (NPY) are of great medical interest and their investigation has only started recently. NPY is a neurotransmitter which is released both by central and peripheral neurons (Lundberg et al., 1982; O'Donohue et al., 1985; Stanley and Leibowitz, 1985). It is part of a family of homologous regulatory peptides, including peptide YY (PYY) and pancreatic polypeptide (Tatemoto et al., 1982; Tatemoto and Mutt, 1980). NPY is highly abundant in the central nervous system where it participates in the control of a wide variety of functions including psychomotor activities, cognitive functions, sexual behaviour, food intake, blood pressure regulation, circadian rhytmicity and neuroendocrine regulation (O'Donohue et al., 1985; Stanley and Leibowitz, 1985). In the peripheral nervous system NPY is associated with sympathetic vascular control and release of catecholamines (Westfall et al., 1990). NPY receptors are members of the G-protein- coupled receptor family and they can be investigated directly by binding studies with radiolabelled NPY or PYY (Dumont et al., 1993; Widdowson and Halaris, 1990). These receptors comprise various subtypes, termed Y$_1$-, Y$_2$-, Y$_3$- receptors, but only one of them, the Y$_1$-receptor, has been cloned without unambiguity (Larhammar et al., 1992). Because of the very limited range of existing NPY receptor subtype-selective ligands and the similar chemical structure of most of them, it is plausible that certain NPY receptor subtypes have escaped identification until now. The above considerations have prompted us to explore the ability of venom preparations from various *Conus* species to interact with these receptors.

Screening studies, involving the binding of radiolabeled neuropeptide Y ([^{3}H]NPY) to its receptor sites in calf brain shed light on a hitherto unexpected property of cone snail venoms (Czerwiec et al., 1996a). Instead of inhibiting the binding of the radioligand (a property that could be attributed to the occupancy of the receptors) the venom of *Conus anemone* was able to increase [^{3}H]NPY binding in a concentration-dependent way. Subsequent experiments indicated that this increase is not related to the presence of receptors and that it could involve binding of [^{3}H]NPY to a component in the venom itself.

Venom preparations from several *Conus* species inhibit [^{3}H]NPY binding to NPY receptors in calf frontal cortex and hippocampus membrane preparations. These regions are particularly rich in Y_1- and Y_2-receptors, respectively. No discrimination between these receptor subtypes was seen for 22 venoms and only a weak discrimination was noticed for the venom of *Conus mercator*. To our surprise, we found that the venom of *Conus anemone* increases the binding of [^{3}H]NPY and that this increase is related to the ability of one of the venom's components to bind the radioligand by itself. The characterization and purification of the involved peptide component of *Conus anemone* venom, termed ANPY-toxin (from *Conus anemone* -NPY), (Czerwiec et al., 1996b) is further detailed below.

Taken together, whereas *Conus* venoms were known to contain peptide components which are capable of interfering with the neurotransmission in their prey organisms or ennemies by interacting with ion channels and various receptors (Fainzilber et al., 1995; Olivera et al., 1990, 1991; Shon et al., 1994; Czerwiec et al., 1989, 1993), the present data constitute the first evidence that such venoms contain components which are capabable of interacting with peptide neurotransmitters.

MATERIALS AND METHODS

Chemicals

N-[propionyl-3H] Neuropeptide Y ([^{3}H]NPY) (80 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). Neuropeptide Y (NPY, porcine), polypeptide Y (PYY, porcine) and the analogs [Leu^{31}, Pro^{34}]NPY (porcine) and NPY-(18-36) (porcine) were from Serva (Heidelberg, Germany). Neuropeptide Y-(1-24) (human), pancreatic polypeptide (avian), pancreatic polypeptide (bovine), secretin (human), Dynorphyn A (porcine), BOC-DAKLI (Bolton Hunter coupled dynorphin A analog kappa ligand) and bovine serum albumin (BSA, Fraction V) were from Sigma (St. Louis, MO, USA). BIBP3226 was from Albany Molecular Research Inc. (Albany, New York, USA). Neuropeptide Y-(25-36) (porcine) was a kind gift from Dr. J. Lundberg (Karolinska Institute, Sweden). All other chemicals were of the highest grade commercially available. Bovine serum albumin (BSA ,Fraction V) was from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available. Biogel P-10 was obtained from BioRad (Richmond, CA, USA). Low molecular weight electrophoresis calibration kit, 8% - 25% gradient PhastGel® media, PhastGel® SDS-buffer strips, PhastGel® Blue-R tablets and PhastGel ™ Silver Kit were obtained from Pharmacia Biotech® (Uppsala, Sweden).

Conus venom preparations

Specimens were life taken: *Conus arenatus, C. aulicus, C. canonicus, C. eburneus* (var. polyglotta*), C. furvus, C. geographus, C. litteratus, C. lividus, C. magus, C. marmoreus, C. mercator, C. miles, C. mustellinus, C. namocanus, C. pennaceus, C. rattus, C. vexillum, C. vitulinus* and *C. virgo* near Cebu, the Philippines; *C. tessulatus* was obtained from the Seychelles, *C. pulcher* near Dakkar, Senegal and *C. anemone* (Fig. 2) from South-West Australia. The gastropods were life taken, frozen, shipped to Brussels in dry ice via air and stored at -20°C until use. The following steps were carried out at 0 to 4°C. The venom ducts of *C. anemone* (Fig. 2), *C. pulcher* and *C. tessulatus* were dissected out of the animals, the venom was squeezed out of the duct and homogenised in 10 volumes 30 mM ammonium acetate (w/v) with a Polytron mixer and sonicated three times for 10 sec in a Soniprep 150 sonicator. Whole ducts were homogenised and sonicated for the other species. Suspensions were centrifugated at 9,000 x g for 10 min. and the resulting supernatants stored at -20°C.
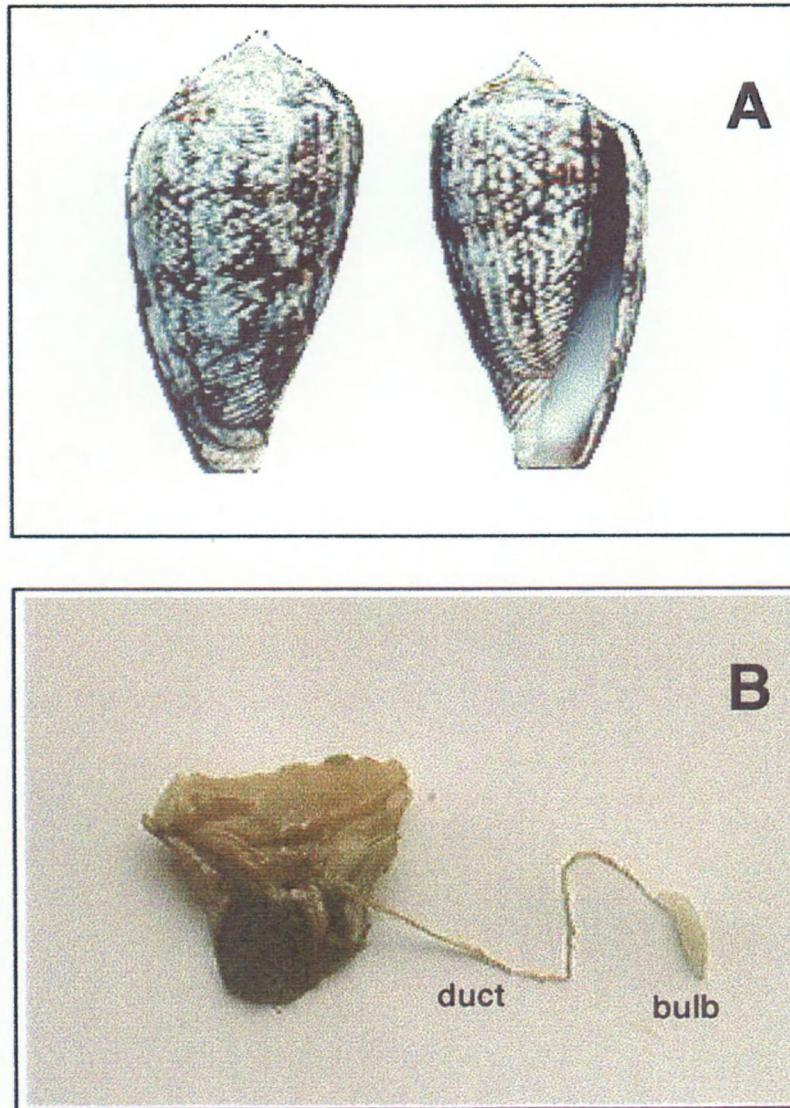
Figure 2: A) Shell of *Conus anemone*, B) animal with dissected venom apparatus

Membrane Preparations.

Calf brains were obtained from a local slaughterhouse within 2 hours post mortem and kept on ice until dissection. Frontal cortex and hippocampus were dissected at 4°C, rapidly frozen in liquid nitrogen and kept at -80°C until further preparation. Frozen rat forebrains were obtained from Iffa Credo (Belgium).

The subsequents steps were caried out at 0-4°C. The brain samples were homogenized with an Ultraturrax and Potter-Elvejhem homogenizer in Krebs-Ringer buffer (137 mM NaCl/ 2.68 mM KCl/ 2.05 mM MgCl$_2$/ 1.80 mM CaCl$_2$/ 20 mM HEPES (pH 7.4)). The homogenate was centrifugated at 30000 x g for 20 min and pellets were resuspended in the same buffer. This procedure was repeated twice and the final pellet was resuspended in Krebs-Ringer buffer containing 10 % glycerol (v/v). The obtained suspensions were stored in Eppendorf tubes in 1 ml batches and kept at - 80°C until use. Batches were thawed and homogenized in Krebs-Ringer buffer and washed by two centrifugations (30000 x g, 20 min) prior to use.

Protein Concentration Determination.

Protein concentrations were determined by a modification of the Sopachem Ultra Sensitive Total Protein Assay, based on the Pyragallol Red-Molybdate complex method (Watanabe, 1986) with BSA as standard.

Purification of [³H]NPY-binding protein from *Conus anemone* venom by gelfiltration chromatography.

Venom was prepared as described above and fractionated over a Biogel P10 column (1.5 cm x 125 cm, elution buffer 50 mM ammonium acetate (pH 7.4)). In a typical experiment 200 μg of protein was loaded on the column and a pressure head of 13 cm/hr was applied. The presence of protein was monitored by absorbance mesaurement at 214 nm and 280 nm using an LKB 2141 variable wavelength monitor. Fractions were lyophilized and resuspended in 50 mM ammonium acetate (pH 7.4) (in 10% of the orginal fraction volume), and assayed for [³H]NPY-binding activity as described below. Fractions containing [³H]NPY-binding activity were pooled and an aliquot was taken for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis).

SDS- Polyacrylamide gelelectrophoresis

Aliquots from crude venom preparation and from pooled active fractions were lyophilized and resuspended in sample buffer containing 10 mM Tris.HCl (pH 6.8)/ 8 mM dithiothreitol/ 2.5% SDS (w/v)/ 10% glycerol (v/v). Standard calibration protein mixture was dissolved in the same

buffer. All samples were boiled for 2 min prior to loading on the gel. Electrophoresis was carried out on 8% - 25% gradient PhastGel® media with PhastGel® SDS-buffer strips using the Phastsystem™ from Pharmacia Biotech®. Coomassie staining was done using PhastGel® Blue-R tablets from Pharmacia Biotech® and silver staining was performed with the PhastGel™ Silver Kit, using the instructions under "Silver staining method optimized for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) with PhastGel® gradient media". Staining solutions, and running and staining conditions were as described in the technical files provided by the company.

Radioligand Binding

Assays were performed in 200 μl Krebs-Ringer buffer containing 0.1% w/v BSA in plastic 96-well plates. Calf or rat brain membrane suspensions (100 μg protein/assay) were incubated for 60 min at 30°C with [^{3}H]NPY (0.1 to 5 nM in saturation experiments and 0.5 nM in competition experiments). Competitor concentrations ranged from 0.01 nM to 1 μM for NPY and analogues and (typically) form 1 to 100 μg/ml for crude venoms. Fractions of the *Conus anemone* venom from the Biogel P10 column were incubated similarly. All assays were performed in plastic 96-well plates in a final volume of 200 μl in Krebs-Ringer buffer containing 0.1 % (w/v) bovine serum albumin.

After incubation, the samples were rapidly filtered through glass fibre filters (Whatman GF/C, incubated in an aquous solution of 0.3 % (v/v) polyethyleneimine 15 min prior to filtration and prewashed with ice-cold Krebs-Ringer buffer) Using a Skatron Cell Harvester. Filters were then washed four times with ice-cold Krebs-Ringer buffer, first for 2 sec and subsequently for one sec. Filters were dried for 10 sec, removed from the harvester and placed in polyethylene scintillation vials with 250 μl 0.1 N NaOH and 3.5 ml scintillation fluid (Optiphase II, LKB). The amount of radioligand on the filters was counted in a liquid scintillation counter.

Centrifugation experiments: *Conus anemone* venom (at indicated protein concentration), rat cortex membrane suspension (typically 100 μg protein/assay) and [^{3}H]NPY (0.5 nM) were incubated in a final volume of 500 μl, using the same conditions as for filtration experiments. After incubation, mixtures were centrifugated for 10 minutes at 30000 g, the pellets were rinsed with 0.5 ml of ice-cold incubation buffer, resuspended in 500 μl 1% Triton X-100 and 400 μl suspension was transferred to scintillation counting vials. 4 ml of Optiphase 'HiSafe' 2 from Wallac (Milton Keynes, UK) was added and radioactivity was counted in a liquid scintillation counter.

Data Analysis

Non-specific binding of [^{3}H]NPY to the membranes as well as to *Conus anemone* venom (10 µg protein/ml) was assessed in the presence of 0.1 µM NPY. This value was substracted from the total binding to yield specific binding. All binding experiments were performed in triplicate and IC_{50}- and K_D-values were calculated by non-linear regression analysis using GraphPad Prism. Values are given as means ± standard error of the mean (S.E.M.).

RESULTS

Identification and characterization of NPY-receptors in calf brain membranes.

Saturation binding experiments with [^{3}H]NPY reveal that calf hippocampus and frontal cortex membranes contain an apparently homogenous class of high affinity sites for this radioligand. The affinity is equal for both regions (K_D = 1.4 nM ± 0.5 nM, nH=1.02 ± 0.03, n = 3) in cortex and (K_D = 0.7 nM ± 0.2 nM, nH= 0.97 ± 0.06, n = 3) in hippocampus but the density of sites is higher in frontal cortex (B_{max} = 434 ± 180 fmol/mg protein) than in hippocampus (B_{max} = 267 ± 50 fmol/mg protein).

In cortex and hippocampus, competition binding experiments with NPY, PYY and NPY-(18-36) result in steep curves and are best analysed in terms of a one site model (Fig.3A and 3B). The K_i-values and, hence, the ranking order of potency of these competitors is identical for both tissues: K_i NPY= K_i PYY< K_i NPY-(18-36) (Table 1). On the other hand, competition curves for the NPY analogue [Leu^{31}, Pro^{34}]NPY are biphasic in both preparations. [Leu^{31}, Pro^{34}]NPY displays high affinity (K_i less than 1 nM) for 80 % ± 1 % of the sites in frontal cortex membranes and for 23 % ± 3 % of the sites in hippocampus membranes (Table 1). The remaining sites display about 1000-fold lower affinity for this competitor in both tissues.

Competitor	CORTEX		HIPPOCAMPUS	
	Ki (nM)	% R	Ki (nM)	% R
NPY			1.05 ± 0.05	100
	0.7 ± 0.1	100		
PYY	1.3 ± 0.1	100	0.7 ± 0.3	100
NPY-(18-36)	29.5 ± 0.5	100	25 ± 0.5	100
[Leu31, Pro34] NPY	H A:0.7 ± 0.2	80 ± 1	0.4 ± 0.1	23 ± 2
	L A:326 ± 27	20 ± 1	590 ± 20	77 ± 2

Table 1. Parameters for NPY-, PYY-, [Leu^{31}, Pro^{34}]NPY- and NPY-(18-36) for competing with [3H]NPY binding in calf frontal cortex and hippocampus membrane preparations. The Ki values and relative amounts of high (HA) and low (LA) affinity sites refer to the competition curves in Fig. 3A and 3B. Values are means and SEM of 3 experiments.

Effects of crude *Conus* venom preparations on [³H]NPY binding.

[³H]NPY binding to calf hippocampus and frontal cortex membranes was tested in the presence of increasing concentrations (typically 1 to 100 μg protein/ml) of crude venom from 23 *Conus* species. The venoms of *Conus pennaceus* and *Conus aulicus* inhibited the binding with high
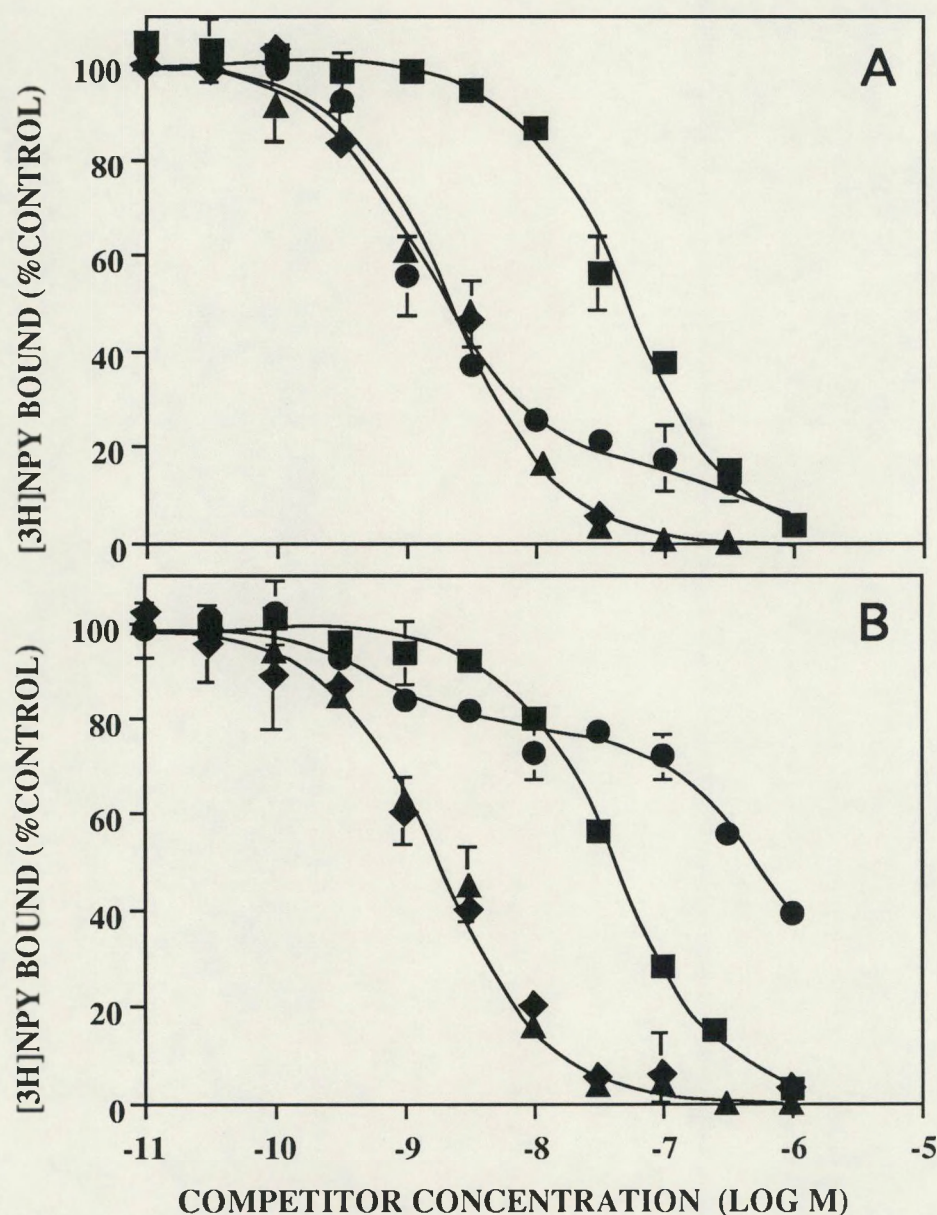

Figure 3: NPY, NPY-(18-36), [Leu³¹, Pro³⁴]NPY and PYY competition binding to NPY receptors in calf cortex and hippocampus. Calf cortex (**A**) and hippocampus (**B**) membrane preparations were incubated with 0.5 nM [³H]NPY and increasing concentrations of the agonists NPY (▲), NPY- (18-36) (■), [Leu³¹, Pro³⁴]NPY (●) and PYY (◆). Binding is experessed in percent control binding, i.e. binding in the presence of buffer only. Table 1 lists the Ki values from the different curves. Values are means and bars are SEM from 3 experiments.

potency (IC_{50} < 5 µg protein/ml) but did not discriminate between the two membrane preparations (Table 2). Several venoms inhibited the binding with low potency (IC_{50} ranging between 10 and 100 µg protein/ml) and did not discriminate between the two membrane preparations (IC_{50}-ratio: ≤ 2, Table 2): *Conus tessulatus, C. marmoreus, C. lividus, C. mercatur, C. pulcher, C. eburneus, C. geografus, C. planorbis, C. magus, C. canonicus, C. vexillum*. A third class of venoms inhibited [^{3}H]NPY binding with low potency in calf frontal

SPECIES	CORTEX (Y1) IC50	HIPPOCAMPUS (Y2) IC50	RETINA (α2) IC50	Y2/Y1	α2/Y1	α2/Y2
C. pennaceus	1.3 ± 0.2	1.4 ± 0.6	-	1	> 77	> 72
C. aulicus	3.4 ± 1	3.4 ± 0.9	18 ± 2	1	5.3	5.3
C. tessulatus	10 ± 4	21 ± 5	18 ± 1	2.1	1.8	0.8
C. marmoreus	17 ± 2.5	11 ± 1	-	0.6	> 6	> 9
C. lividus	18 ± 2	24 ± 1.5	-	1.3	> 6	> 4
C. mercator	24 ± 9	150 ± 24	102 ± 14	6	4.2	0.7
C. pulcher	31 ± 11	19 ± 6	-	0.6	> 3	> 5
C. eburneus	34 ± 4.5	64 ± 19	18 ± 2.3	1.9	0.5	0.3
C. vitulinus	38 ± 4	-	41 ± 6	> 2	1	< 0.4
C. geografus	43 ± 8	31 ± 4	18 ± 0.7	0.7	0.4	0.5
C. magus	55 ± 1	51 ± 3	29 ± 6	0.9	0.5	0.5
C. canonicus	56 ± 14	110 ± 6	57 ± 1.5	2	1	0.5
C. rattus	56 ± 20	-	-	> 2	> 2	-
C. vexillum	62 ± 3	79 ± 4	52 ± 9	1.3	0.8	0.6
C. arenatus	-	-	5 ± 1	-	< 0.05	< 0.05
C. furvus	-	-	-	-	-	-
C. litteratus	-	-	-	-	-	-
C. miles	-	-	-	-	-	-
C. mustellinus	-	-	-	-	-	-
C. namocanus	-	-	-	-	-	-
C. virgo	-	-	-	-	-	-

Table 2. Parameters for crude *Conus* venom preparations for competing with [3H]NPY binding in calf frontal cortex (80 % Y_1, 20 % Y_2) and hippocampus (23 % Y_1, 77 % Y_2) and for [^{3}H]idazoxan binding in retina membrane preparations (α_2-adrenergic receptors, Convents et al., 1987). IC_{50} -values are given in µg protein/ml. Y_2/Y_1, α_2/Y_1 and α_2/Y_2 refers to the ratio of IC_{50}-values obtained for the respective receptors. Values are means and SEM of 3 experiments.

- : IC_{50} > 100 µg protein/ml.

cortex and showed even ≥ 2-fold lower potency in calf hippocampus: *Conus mercator, C. vitulinus, C. rattus* (Table 2). The IC_{50} of the venom of *Conus mercator* for hippocampus membranes was found to be 6 times lower than for cortex membranes but the two other venoms were too weak to allow the determination of their IC_{50} for hippocampus membranes. A fourth class of venoms were poor inhibitors (IC_{50} > 100 μg protein/ml) for both membrane preparations: *Conus arenatus, C. furvus, C litteratus, C. miles, C mustelinus, C. namocanus* and *C. virgo* (Table 2).

The behaviour of the venom of *Conus anemone* was completely unexpected; it increased the binding of [³H]NPY up to 5 times (Fig 4 A and B). This increase was dose-dependent with a half-maximal effect at 15 ± 1 μg protein/ml and 18 ± 0.5 μg protein/ml in the presence of cortex and hippocampus membranes, respectively. Competition binding studies with NPY, PYY and the analogues, [Leu³¹, Pro³⁴]NPY and NPY-(18-36) were carried out to find out whether the increased binding involved Y_1-receptors, Y_2-receptors or even unrelated sites. For this purpose venom from *Conus anemone* (in a final concentration of 25 μg protein/ml, increasing the binding up to 3- fold) was included in the competition assay in cortex or hippocampus membrane suspensions. Characteristics of the increased [³H]NPY binding clearly differed from those of the [³H]NPY binding in control membranes (Fig. 4 A and B, insert). [Leu³¹, Pro³⁴]NPY, NPY-(18-36), PYY and NPY inhibition curves were all steep and analysed according to a one site model; IC_{50}-values were comparable and the potency ranking order was identical for both membrane preparations: PYY < [Leu³¹, Pro³⁴]NPY ≈ NPY-(18-36) ≈ NPY (Table 3).

Characterization of the [³H]NPY binding to *Conus anemone* venom.

Control experiments, in which membranes were omitted, revealed that the venom-increased the binding of [³H]NPY to the same extend as in the presence of membranes. Incubation of crude *Conus anemone* venom preparation with 0.5 nM [³H]NPY for 1 h at 30°C and subsequent vacuum-filtration of the mixture over polyethyleneimine (0.3 % v/v)-pretreated glass fiber filters resulted in the retention of a substantial amount of the radioligand by the filters (Table 4). About 80 % of this binding could be displaced by addition of 0.1 μM unlabeled NPY, and since it was not observed in the absence of venom it was defined as specific binding of [³H]NPY to one or more of the venom's components. This specific binding could not be detected when the filters had not been pretreated with polyethyleneimine (Table 4).
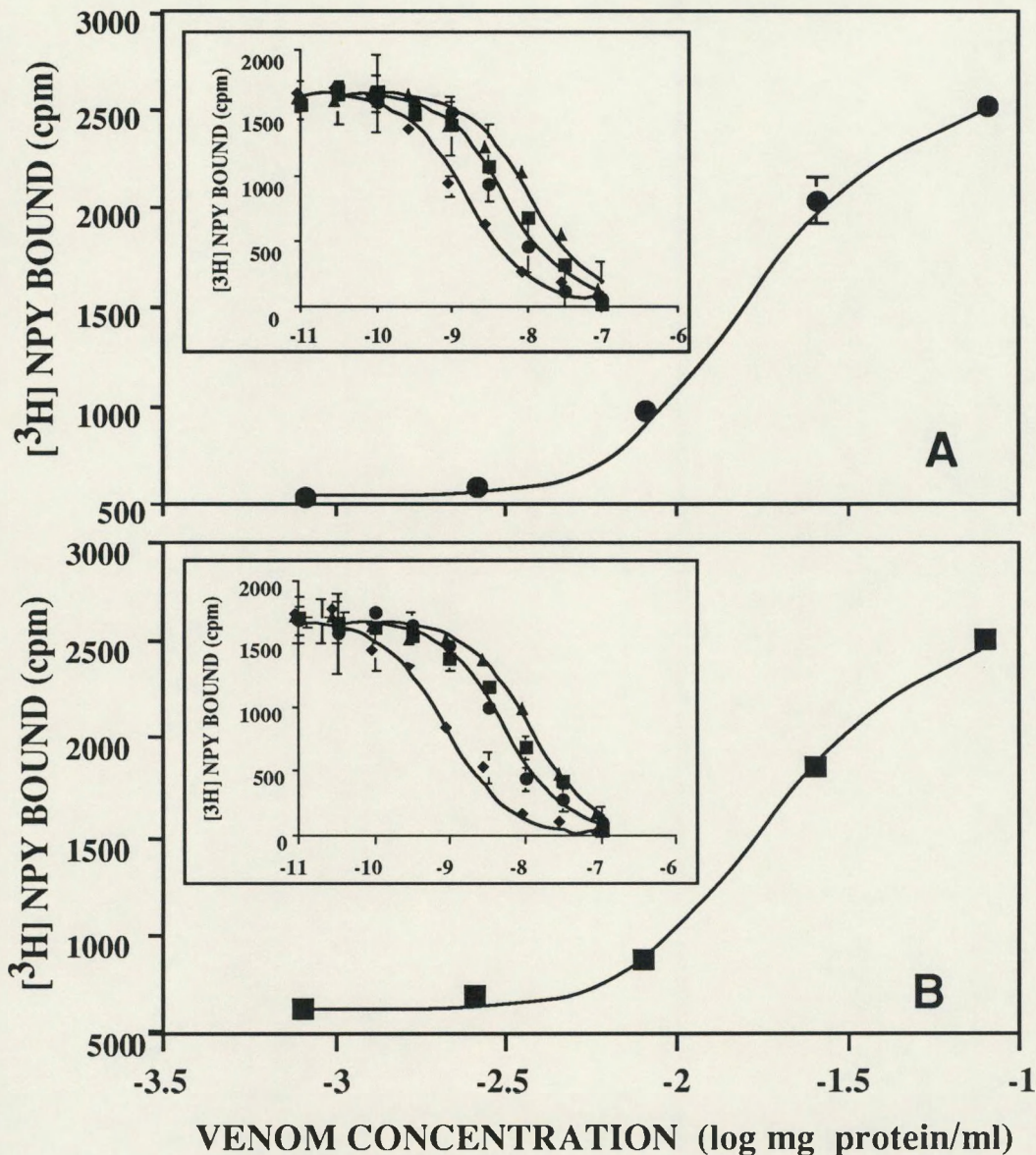
Figure 4: Increase of [³H]NPY binding mediated by *C.anemone* venom. Membrane preparations form calf cortex (80 % Y_1, 20 % Y_2) (A) and hippocampus (23 % Y_1, 77 % Y_2) (B) were incubated with 0.5 nM [³H]NPY and increasing concentrations of *C. anemone* venom. Binding is given as total cpm. Values are means and bars are SEM from 3 experiments. Inserts: competition with the agonists NPY (▲), NPY- (18-36) (■), [Leu³¹, Pro³⁴]NPY (●) and PYY (◆)) on increased [³H]NPY binding in the presence of *C. anemone* venom. Calf cortex (A) and hippocampus (B) membrane preparations were incubated with 0.5 nM [³H]NPY, *C. anemone* venom preparation (final concentration 25 µg protein/ml, increasing the binding up to 3-fold) and increasing concentrations of competitors. Increased specific binding is given in cpm and was calculated by subtracting non specific binding (measured in the presence of 0.1 µM NPY) from control binding (measured in the presence of *C. anemone* venom and buffer alone). Table 3 lists the IC_{50} values and Hill coefficients from the different curves. Values are means and S.D. from two experiments.

Competitor	CORTEX		HIPPOCAMPUS	
	IC50 (nM)	nHill	IC50 (nM)	nHill
NPY	13 ± 5	0.96 ± 0.05	11 ± 3	0.84 ± 0.09
PYY	1.8 ± 0.1	0.81 ± 0.01	0.85 ± 0.05	0.82 ± 0.01
NPY-(18-36)	5.9 ± 0.5	0.88 ± 0.08	7.0 ± 0.1	0.72 ± 0.05
[Leu31, Pro34]NPY	5.4 ± 0.9	1.1 ± 0.9	5.3 ± 1	1.0 ± 0.7

Table 3. Parameters for NPY-, PYY-, [Leu^{31}, Pro^{31}]NPY- and NPY-(18-36) for competing with [^{3}H]NPY binding to a mixture of venom from *C. anemone* (final concentration 25 µg protein/ml) and frontal cortex or hippocampus membrane preparations. IC$_{50}$ -values and Hill coefficients refer to the competition curves in Fig. 4A and 4B. Values are means and SD from 2 experiments.

Venom concentration (µg protein/ml)	Total binding (cpm)	Non specific binding (cpm)
Non-pretreated filters		
0	850 ± 61	812 ± 52
10	702 ± 7	N.D.
20	613 ± 32	N.D.
30	507 ± 10	N.D.
PEI-pretreated filters		
0	412 ± 25	441 ± 47
10	1162 ± 8	N.D.
20	1728 ± 52	N.D.
30	2103 ± 28	N.D.

Table 4: Binding of [^{3}H]NPY to a component in *C. anemone* venom. [^{3}H]NPY (0.5 nM) is incubated for 60 min at 30°C with or without *C. anemone* venom (at the indicated concentrations) and incubation is stopped by vacuum-filtration over Whatmann GF/C filters. Filters were not pretreated or pretreated with (0.3% v/v) polyethyleneimine. Non specific binding was measured in the presence of 0.1 µM unlabeled NPY. Values refer to radioactivity (cpm) retained on the filter and are means and S.E.M. of three experiments. N.D.: not determined.

Saturation binding experiments, wherein a constant concentration (10 μg protein/ml) of venom was incubated with increasing concentrations of [^{3}H]NPY showed that the specific binding occurs with high affinity and is saturable (Fig. 5). Scatchard plots (Fig. 5, insert) are linear indicating the presence of a homogeneous population of binding sites. The saturation binding parameters (K_D = 2.95 ± 0.20 nM and B_{max} = 15.2 ± 0.5 pmol/mg protein) were determined by non-linear regressin analysis. In contrast, the non-specific binding (determined in the presence of 0.1 μM unlabeled NPY increased proportionally to the [^{3}H]NPY concentration.
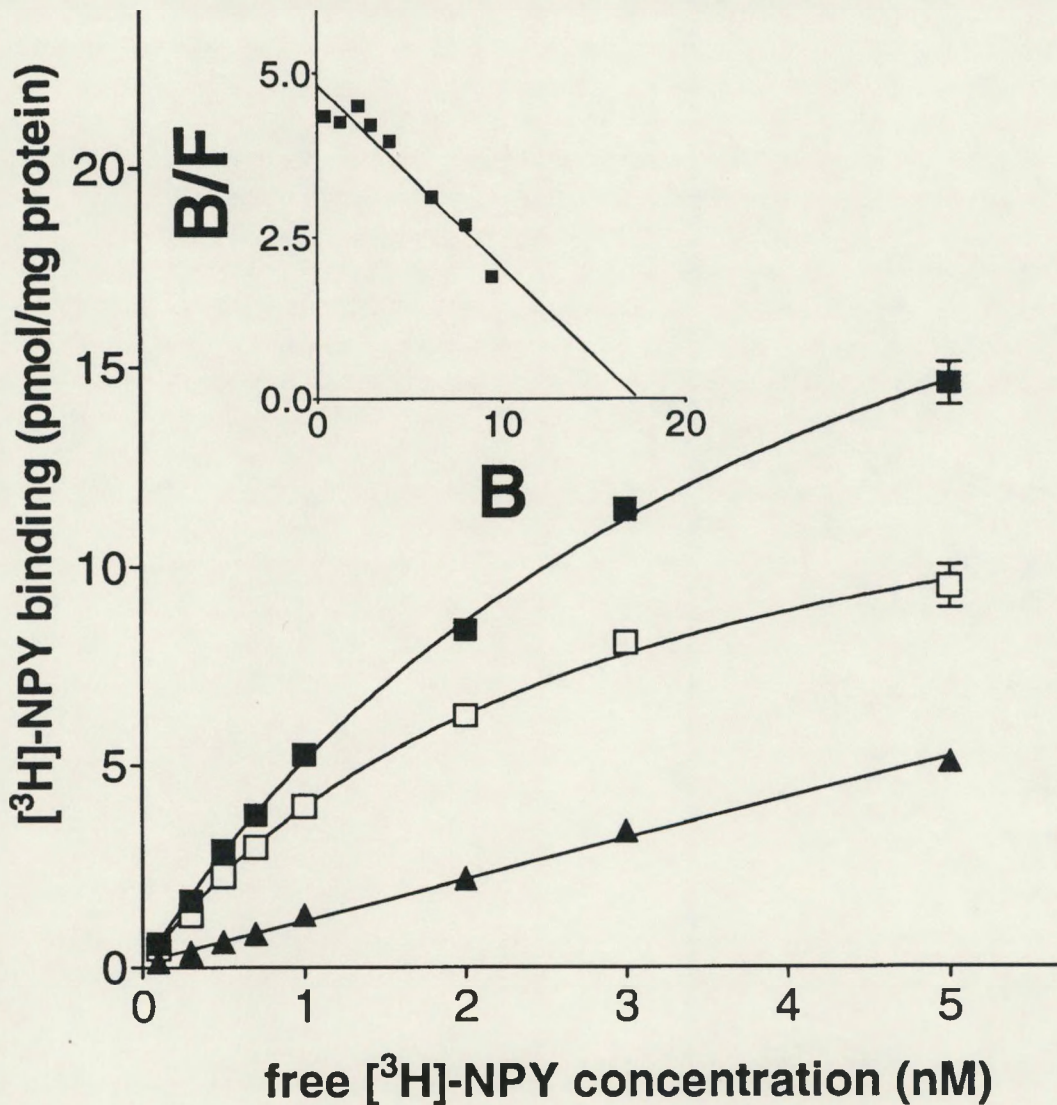

Figure 5: Saturation binding of [^{3}H]NPY to *C. anemone* venom. Venom (10 μg protein/ml) was incubated for 60 min at 30°C with increasing concentrations of [^{3}H]NPY (0.1 to 5 nM) and vacuum-filtered over polyethyleneimine (0.3% v/v) pretreated Whatmann GF/C filters. Specific binding (□) was obtained by subtracting non specific binding (in presence of 0.1 μM NPY) (▲) from total binding (■). The curves represent means of three expriments. The K_D- and B_{max}-value from the [^{3}H]NPY binding is given in Results. *Insert*: Scatchard plot of the data saturation binding data. Curves are means from three experiments.

Specific [^{3}H]NPY binding to *Conus anemone* venom could be completely displaced with nanomolar affinity by unlabeled human and porcine NPY, porcine [Leu^{31}, Pro^{34}]NPY, porcine PYY and avian and bovine pancreatic polypeptide (Table 5). All competition curves had Hill slopes of unity (Data not shown). Similar high affinity displacement was seen with the (18-36) and (25-36) C-terminal fragments of NPY but not with the (1-24) N-terminal fragment of human NPY. No displacement was found with peptides which are unrelated to the pancreatic polypeptide-fold family such as human secretin, porcine dynorphin A and Boc-DAKLI (Bolton Hunter coupled dynorphin A analog kappa ligand) (Table 5), nor with the non-peptide NPY receptor antagonist BIBP3226 (Data not shown).

A substantial proportion of the [^{3}H]NPY binding to rat cortex membranes could be displaced with unlabeled NPY (IC_{50} = 5 nM) and about 80 % of this specific (i.e. NPY-displaceable) binding displayed high affinity for the Y_1-selective antagonist BIBP3226 (IC_{50} = 6.3 nM, Fig. 7). A similar predominance of Y_1-receptors has also been reported by others by using less

Peptide	Amino acid sequence	IC50 (nM)
human NPY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY	8.7 ± 1.0
porcine NPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY	9.3 ± 1.0
human NPY-(1-24)	YPSKPDNPGEDAPAEDMARYYSAL---------------------	>1000
NPY-(18-36)	---------------------------------- ARYYSALRHYINLITRQRY	29.5 ± 1.0
NPY-(25-36) porcine	--RHYINLITRQRY	5.5 ± 0.9
[Leu^{31},Pro^{34}]NPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLLTRPRY	2.6 ± 1.0
porcine PP	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY	7.4 ± 1.1
avian PP	GPSQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	1.5± 0.15
bovine PP	APLEPEYPGDDATPEQMAQYAAELRRYINMLTRPRY	2.3 ± 0.9
human secretin	HSDGTFTSELSRLREGARLQRLLQGLV	> 1000
porcine dynorphin A	YGGFLRRIRPKLKWDNQ	> 1000
Boc-DAKLI	N-t-Boc-YGGFLRRIRPRLRG-5-aminopentylamide	> 1000

Table 5: Competition binding parameters of various peptides for the specific [^{3}H]NPY binding to *C. anemone* venom. Amino acid sequences of tested peptides is given together with the respective IC_{50}-values. Mixtures of [^{3}H]NPY (0.5 nM) and *C. anemone* venom (25 μg protein/ml) are incubated for 60 min at 30°C with increasing concentrations of competitor (typically between 0.01 nM and 1 μM) and incubation is stopped by vacuum-filtration over polyethyleneimine (0.3% v/v) pretreated Whatmann GF/C filters.
IC_{50}-values and S.E.M. are calculated using the GraphPad Prism program. Values are means and S.E.M. from three experiments.
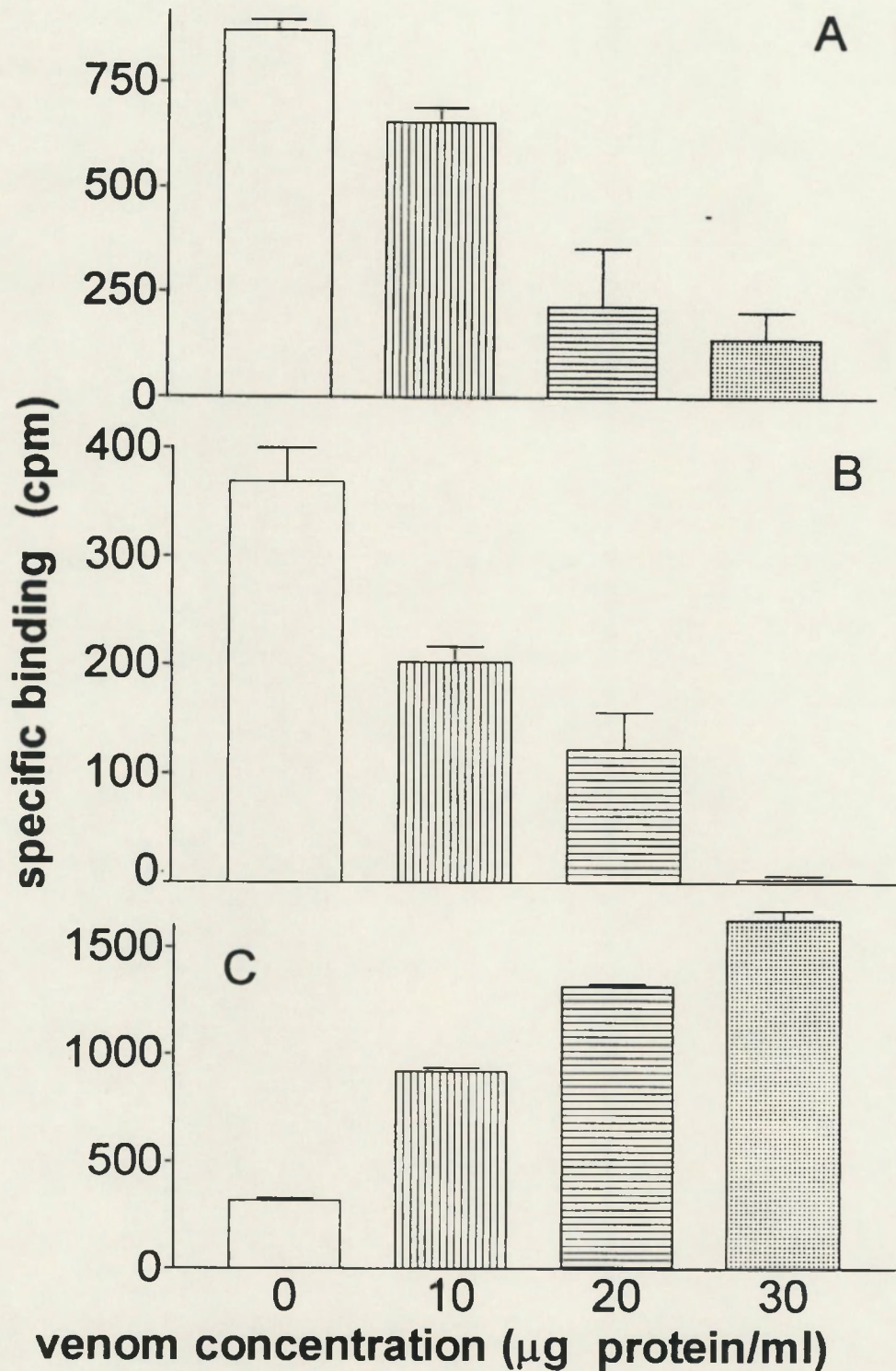
Figure 6: Effect of *C. anemone* venom on [³H]NPY binding to rat cortex membranes. Rat cortex membranes were incubated with 0.5 nM [³H]NPY for 60 min at 30°C, in the absence or presence of the indicated concentrations of venom. Mixtures were then subjected to centrifugation (**A**) and radioactivity of the pellet measured, filtered over non-pretreated Whatmann GF/C filters (**B**) or filters pretreated with 0.3% (v/v) polyethylene imine (**C**). Non specific binding was measured in the presence of 0.1 µM unlabeled NPY. Bars represent means of 3 experiments and error bars represent S.E.M. values.

Purification of the [³H]NPY binding protein from *Conus anemone* venom.

selective compounds (Dumont et al., 1993). When added to this mixture, the venom of *Conus anemone* produced a concentration -dependent increase in specific binding of [³H]NPY when free and bound radioligand was separated by filtration on polyethyleneimine-treated filters (Fig. 6 C). This increase may be attributed to the trapping of venom-bound [³H]NPY by the filters. Specific binding of [³H]NPY to its receptors still occured when separations were performed by using non-pretreated filters or by centrifugation but, under these conditions, the venom of *Conus anemone* produced a concentration -dependent decrease in binding (Fig. 6 A and B).

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) from crude *Conus anemone* extract reveals that some high molecular weight components are present and that a fair amount of low molecular weight components is detected by silver staining (Fig. 9 A). The major component of the venom has a molecular weight of 18.5 kDa (Fig. 9).
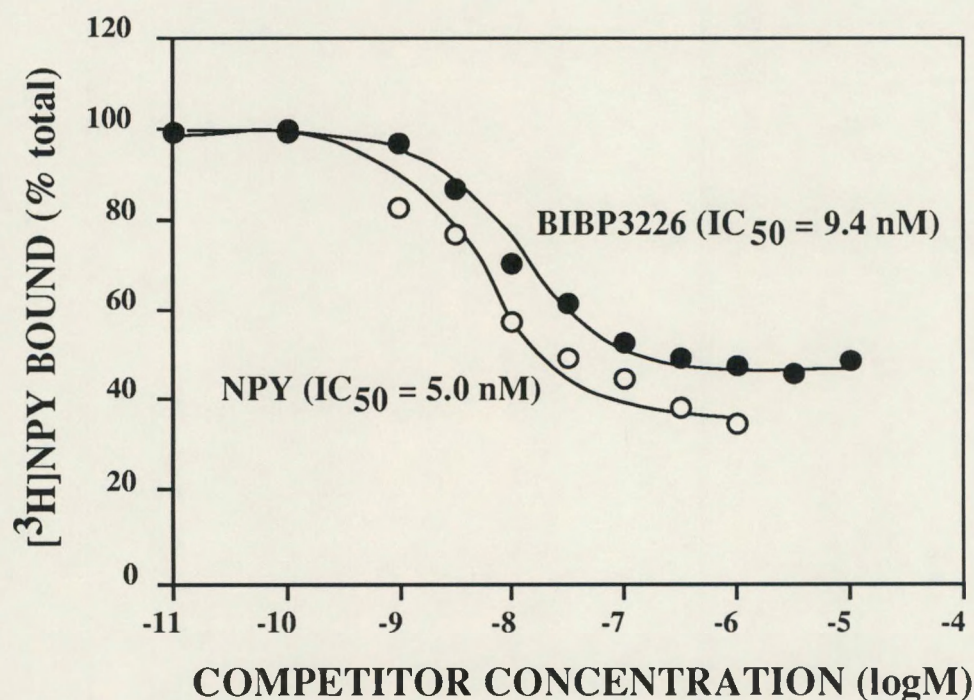
Figure 7: Competition binding on NPY receptors from rat cortex membranes. Membranes were incubated with [³H]NPY (0.5 nM) and increasing concentrations of unlabeled NPY (O) and the non-peptide selective Y1 receptor antagonist BIBP3226 (●). Data were obtained in one representative experiment with each point determined in triplicate.
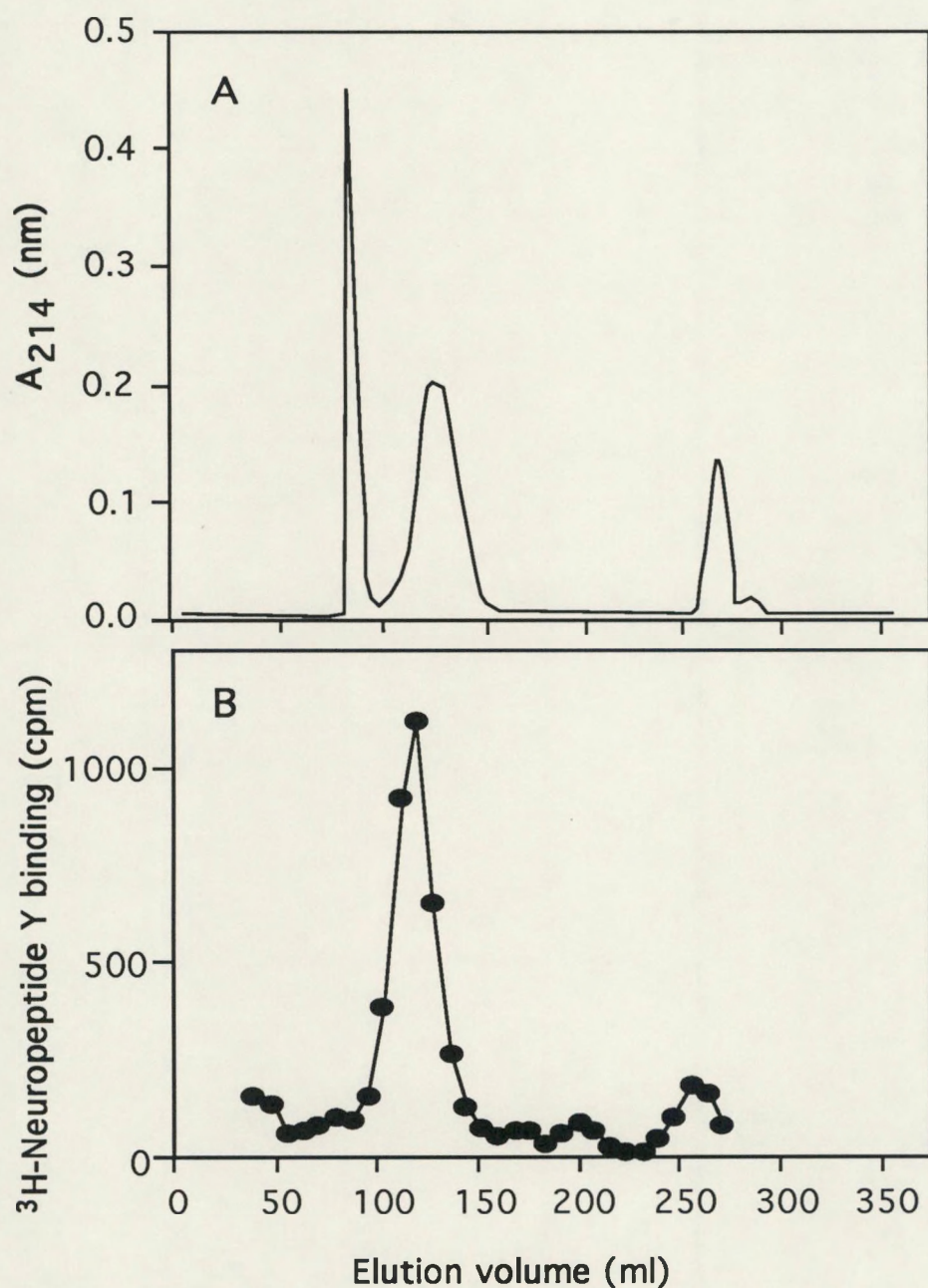
Figure 8: A. Gelfiltration chromatogram of *C. anemone* venom fractionation experiment. A Biogel P10 column (1.5 cm x 125 cm, elution buffer 50 mM ammonium acetate (pH 7.5)) was loaded with 200 μg of protein dissolved in elution buffer. Fractions of 10 ml were collected and lyophilized, resuspended in elution buffer (10 % of the original fraction volume) and tested for [^{3}H]NPY binding activity as described under Methods. Three major peaks are detected by absorbance measurement at 214 nm. **B.** Specific binding of [^{3}H]NPY (0.5 nM) above base-line values (in the absence of fractionated material). Other fractionation experiments gave identical results.

During fractionation of the crude *Conus anemone* venom over a Biogel P10 gelfiltration resin (fractionation range 1.5 - 20 kDa), three major absorbance peaks are detetected (at 214 nm (Fig. 8 A) and 280 nm (Data not shown)). The elution volume of the first peak coincides with the void volume of the column and contains material with a MW > 20 kDa. The $[^3H]$NPY binding activity was confined to the second peak and was insignificant in any of the other fractions (Fig. 8 B). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) of material from the second peak shows the presene of a single band (as revealed by using Coomassie or Silver staining) that migrates in an identical way as the 18.5 kDa band in the crude extract (Fig. 9 A and B).
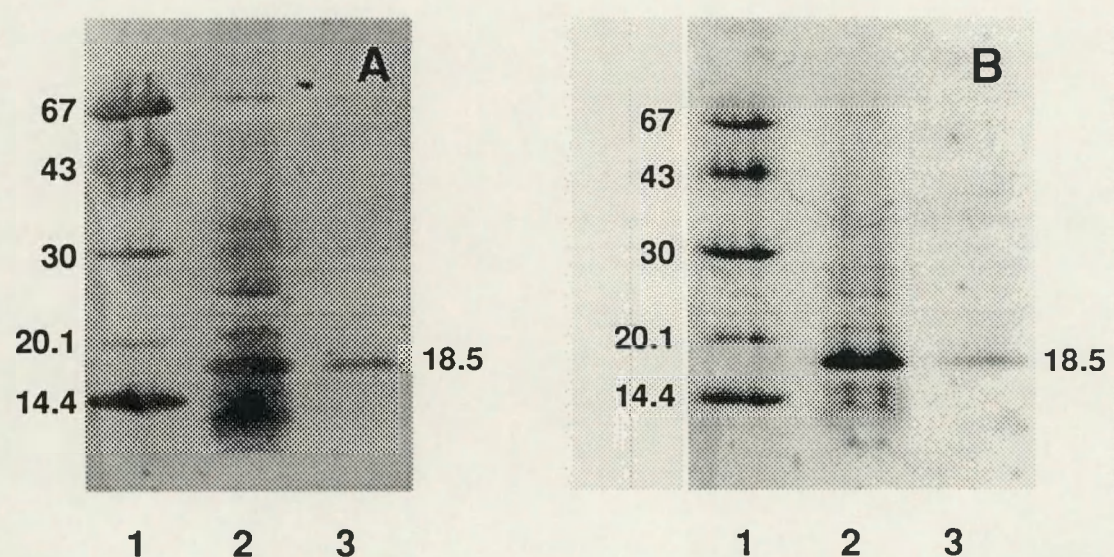

Figure 9: SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) of crude *C. anemone* venom and $[^3H]$NPY-binding protein. Standard protein mixture (lane 1), crude venom preparation (lane 2) and $[^3H]$NPY-binding protein (lane 3) were loaded on gradient gels (8%-25%). Identical samples (typically 1-5 µg protein) were loaded on two gels that were run in parallel in a Phastsystem. Protein bands were visualized using Silver (A) and Coomassie Blue staining (B). Standard protein mixture contained bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumine (14.4 kDa). Samples loaded in lanes 2 and 3 were from the experiment described under Fig. 8.

DISCUSSION

Identification and characterization of NPY-receptors in calf brain membranes.

Based on the ranking order of potency of NPY and certain peptide analogues, NPY receptors are currently classified into three subtypes: Y_1-, Y_2-, and Y_3-receptors. Whereas NPY does not discriminate between the three subtypes, PYY is only recognised with high affinity by the Y_1- and Y_2-receptors. C-terminal fragments of NPY such as NPY-(13-36) and NPY-(18-36) display poor affinity for the Y_1- receptors whereas NPY-analogues wherein Ile at position 31 and Gly at position 34 are substituted by Leu and Pro, respectively, (i.e. $[Pro^{34}]$NPY and $[Leu^{31}, Pro^{34}]$NPY) have greatly reduced affinity for the Y_2- receptors (Boubik et al., 1989; Fuhlendorff et al., 1990a; Grundemar and Hakanson, 1994; Sheikh et al., 1989). Synthetic non-peptide antagonists with high selectivity for the Y_1-receptors have recently been developed (Rudolf et al., 1994; Serradeil-Le Gal et al., 1995).

NPY receptors have already been studied in the central nervous system of rat, pig and man. From these studies, it appears that the three decribed subtypes are present in the central nervous system and that their abundance varies from one region to another and, for the same brain region, from one species to another (Busch-Sorensen et al., 1989; Dumont et al., 1990, 1993; Widdowson and Halaris, 1990). Y_2-receptors clearly predominate in the hippocampus of all investigated species. Y_1-receptors, on the other hand, predominate in frontal cortex of rat and pig, but their concentration is appreciably lower than that of the Y_2-receptors in the human frontal cortex. Less is known about the Y_3-receptor subtype that has been identified in the brainstem from rat (Grundemar et al., 1991a,b). In the present study, we found that calf frontal cortex and hippocampus membranes contain NPY receptors as well. They show high, nanomolar affinity for the radioligand $[^3H]$NPY and their densities are higher than those reported for human and rat cortex (Widdowson and Halaris, 1990), comparable to that reported for pig cortex and lower than for pig hippocampus (Busch-Sorensen et al., 1989).

Competition binding studies indicate that PYY displaces the binding of $[^3H]$NPY to calf cortex and hippocampus membranes to the same extend and with the same potency as NPY itself (Fig. 3). Since Y_3- receptors are characterised by their very low affinity for PYY (Grundemar et al., 1991a,b) our data indicate that Y_3- receptors are not present at detectable levels in these brain regions. The Y_1- and Y_2- receptor subtypes are usually discriminated from each other on basis of their difference in affinity for synthetic NPY analogues such as $[Leu^{31}, Pro^{34}]$NPY which is Y_1-selective and C-terminal fragments (e.g. NPY-(18-36)) which act preferentially on Y_2-receptors.

The competition binding data with $[Leu^{31}, Pro^{34}]NPY$ are clearly biphasic in both membrane preparations. The high affinity sites, represent about 80 % of the labelled sites in cortex membranes and about 23 % in hippocampus membranes (Fig. 3). This high affinity sites have, in both tissues, a ranking order of potency that fits with a Y_1 - subtype: NPY = PYY = $[Leu^{31}, Pro^{34}]NPY$ > NPY-(18-36). The pharmocological profile of the low affinity sites is consistent with that of a Y_2: NPY = PYY > NPY-(18-36) > $[Leu^{31}, Pro^{34}]NPY$. It can thus be concluded that both membrane preparations contain Y_1 - as well as Y_2 - receptors and that their ratio is about 4 to 1 in calf frontal cortex membranes and 1 to 3 in calf hippocampus membranes. These relative amounts are comparable to those reported for rat and pig cortex and hippocampus (Dumont et al., 1993).

Competition curves with the C-terminal fragment NPY-(18-36) are steep and the potency of this peptide is about 30 times lower than for NPY in both membrane preparations. These findings suggest that, at least in calf membranes, NPY-(18-36) does not distinguish between the Y_1- and Y_2- receptor subtypes on its own and, hence, is not suitable for discriminatory purposes when used as the only competitor.

Effects of crude *Conus* venom preparations on $[^3H]NPY$ binding.

The second part of this study, dealing with the effect of *Conus* venoms on $[^3H]NPY$ binding to calf cortex and hippocampus membranes, provided two noteworthy observations. Firstly, from the 23 venoms tested, 17 were able to inhibit the binding of $[^3H]NPY$ (by at least 50 % at 100 μg protein/ml) to at least one of the membrane preparations (Table 2). Yet, these venoms showed marked differences in potency and only two of them (from *Conus pennaceus* and *Conus aulicus*) could inhibit the binding with IC_{50} values below 5 μg protein/ml. Both venom preparations were equipotent in calf cortex and hippocampus membranes and, hence did not present NPY receptor- subtype selectivity. From the other active venoms, none produced preferential inhibition in hippocampus membranes. For calf cortex membranes, only the venoms of *Conus mercator*, *Conus vitulinus* and *Conus attus* showed some, albeit limited selectivity: 6-fold for the venom of *Conus mercator* (no values could be calculated for the other venoms because IC_{50} values in hippocampus were too low). Although the tested *Conus* venoms display no or only limited NPY- receptor subtype selectivity, some of them are quite effective at differentiating the NPY receptors from other G- protein coupled receptors such as the α_2-adrenergic receptor. This is clearly illustrated for the venoms of *Conus pennaceus* and *Conus aulicus* , which inhibit the binding of the α_2-adrenergic antagonist $[^3H]RX781094$ to its

receptors in calf retina membranes with appreciably lower potency then the binding of [³H]NPY in cortex and hippocampus; about 5 times for the venom of *Conus aulicus* and over 70 times for the venom of *Conus pennaceus*. Alternatively, the venom of *Conus arenatus* is a quite potent inhibitor of the binding to α_2-adrenergic receptors and more than 20 times less potent for the NPY receptors in cortex and striatum (Table 2). Since certain of the tested venoms can readily differentiate α_2-adrenergic receptors from NPY receptors, it could be deduced that the limited Y_1- versus Y_2- receptor selectivity of these toxins is due to the structural similarity between these receptor subtypes. The validity of this deduction may be verified by comparing the amino acid sequences of the two receptor subtypes but, since only the Y_1- receptor has been cloned without ambiguity (Larhammar et al., 1992), such verification will have to await cloning data for the Y_2-receptor. Isolation of the venom components which are responsible for decreasing the binding of [³H]NPY to Y_1- and Y_2- receptors (in the venoms of *Conus pennaceus* and *Conus aulicus*) and for the direct interaction with the radioligand receptors (in the venom of *Conus anemone*) could also allow us to gain further insight in their molecular structure and properties.

[³H]NPY binding to *Conus anemone* venom.

Quite unexpectedly, it was found that the venom from *Conus anemone* contains one or more components which are capable to increase the binding of [³H]NPY as assessed by our filtration binding method. Since this increase occurs independently from the presence of membranes, it is likely that these venom components interact with [³H]NPY itself and then get trapped by the glass fibre filters. An alternative explanation such as the precipitation of [³H]NPY is, in our opinion, much less likely because of the very low concentration of this radioligand (0.5 nM) in the assay. Interestingly, these venom components do not have a pharmacological profile that would classify them as one of the known NPY receptor subtypes (Table 3), so that they seem to be unrelated to any of the known NPY receptor subtypes. Binding of NPY- and some related peptides to the involved peptide component of the *Conus anemone* venom , further denominated as "ANPY" toxin, obeys specific structural requirements.

The ANPY toxin displays high affinity for NPY (K_D of 3 nM by saturation binding, and IC_{50} of 8.7 nM by competition binding, Fig. 5 and Table 4) as well as for other relatives of the pancreatic polypeptide family such as peptide YY and avian and bovine pancreatic polypeptide (Table 5). Competition binding experiments with NPY fragments reveal that the N-terminal fragment NPY-(1-24) is devoid of binding activity while the C-terminal fragments NPY-(18-36) and NPY-(25-36) are nearly as active as the native peptide. These data clearly show that the C-terminal portion of NPY is crucial for the binding to the toxin. In the same line, it has been

shown that C-terminal fragments of NPY still display reasonable affinity for Y_2- receptors (Boublik et al., 1989; Sheikh et al., 1989) but the structural freedom is much more limited than for the toxin. Indeed, very short fragments such as NPY-(25-36) display already over 10,000 times lower affinity for the Y_2-receptors in rat hippocampus as compared to NPY (Danho et al., 1988) . In this line, it is also interesting to notice that the affinity of NPY-(25-36) for the Y_2-receptors is about 500 times lower than for the toxin. This distinction suggests that the binding epitope on the toxin is quite different from those on the NPY receptors.

Spatial aspects of NPY have been investigated in detail by various techniques and the peptide is determined to possess a type II polyproline helix (residues 1-8) and an amphiphilic α-type helix (residues 15-32) which are connected by a type II β-turn (Glover et al., 1985; McKerell, 1988; Minakata et al., 1989; Schwartz et al., 1990) and closely packed together through hydrophobic interactions (Allen et al., 1987) . This confers a stable structure to NPY wherein positively charged groups reside in the combined C- and N-terminal epitope and negatively charged acid residues in the turn region of the pancreatic polypeptide-fold (Schwartz et al., 1990). Because of this clear-cut spacial segregation of the positively and negatively charged groups on the NPY molecule, it is plausible that NPY (and its relatives of the pancreatic polypeptide family) is bound via its positively charged moiety to the toxin. Such electrostatic interactions could explain the strong binding of the positively charged C-terminal fragments of NPY as compared to the very weak affinity of the negatively charged (1-24) N-terminal fragment. NPY-(25-36), the shortest of the active NPY fragments that was tested, contains three arginine residues (at positions 25, 33 and 35) and, as this fragment still displays α-helicity (Jung et al., 1991), their spatial arrangement could be similar to that in the full NPY molecule. In this context, NPY has also been reported to interact with G proteins, inducing histamine release from rat peritoneal mast cells, and the role of the net positive charges of NPY in this process has clearly been evidenced (Fuhlendorff et al., 1990b; Mousli et al., 1994a, 1994b, 1995) . However, since very little is known about the molecular mechanisms which are involved in guiding the NPY molecule to its intracellular target, the G protein, it is unclear whether the positve charge of NPY (and its C-terminal fragments) is required for the recognition by the cell membrane, its transport through the membrane or its recognition by the G protein (Mousli et al., 1994a, 1994b) .

Yet, a model involving merely electrostatic interactions between the positively charged N-terminal epitope of NPY and a negatively charged moiety on the toxin appears to be too simple to explain the NPY- toxin interaction. Indeed, it does not explain why peptides of the pancreatic polypeptide family with an arginine residue at position 25 (NPY, PYY), arginine residues at positions 25 and 26 (bovine pancreatic polypeptide) and only uncharged valine residues at positions 25 and 26 (avian pancreatic polypeptide) have nearly the same affinity for the toxin (Table 5). These peptides have arginine residues at positions 33 and 35 in common but an Arg-Xxx-Arg sequence does not seem to be sufficient to confer high affinity since this sequence is

also present in peptides (human secretin, porcine dynorphin A and Boc-DAKLI (Bolton Hunter coupled dynorphin A analog kappa ligand)) that do not displace [^{3}H]NPY binding from the toxin (Table 5). The active NPY-related peptides all have a C-terminal Tyr residue, but the Arg-Tyr sequence is not sufficient for binding to the toxin neither, since BIBP3226, a Y_1-receptor antagonist which contains such sequence (Rudolf et al., 1994) , is inactive (Data not shown). Taken together, the present competition binding data indicate that the high affinity binding of NPY and its analogs to the toxin may be related to a consensus sequence that is located in the C-terminal portion and is more complex than the Arg-Xxx-Arg and Arg-Tyr combinations. Investigation of the binding activity of even shorter C-terminal fragments (such as NPY-(27-36)) as well as of modified fragments might provide more precise information about the nature and location of the amino acids of NPY which are involved in the binding to the toxin.

Finally, when the filtration technique is used to measure the binding of [^{3}H]NPY to its receptors in membrane preparations, the glass fibre filters are routinely pretreated with positively charged polyethyleneimine to reduce the extend of non-specific binding of the radioligand to the filters. This pretreatment appears to be essential for retaining the [^{3}H]NPY-toxin complexes (Table 4) and it is therefore likely that ionic interactions between the positively charged filters and a negatively charged moiety of the toxin are involved.

Purification of the ANPY toxin from *Conus anemone* venom.

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) of crude *Conus anemone* venom shows that it contains several polypeptide components with high molecular weight and that one of them (18.5 kDa) is particularly abundant. When the crude venom was subjected to gelfiltration chromatography over a column with a fractionation range of 1.5 - 20 kDa, the [^{3}H]NPY-binding activity was found to elute in a single peak, immediately after the void volume (Fig. 8 B). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gelelectrophoresis) of the active fraction revealed a substantial enrichment of the 18.5 kDa polypeptide (Fig. 9). It is thus very likely that the ANPY toxin corresponds to this polypeptide.

Physiological role of the "ANPY" toxin.

At present, we can only speculate about the potential physiological role of the "ANPY" toxin. It seems to be a major constituent of the venom (up to 30% of total protein in some preparations) and association with preying or defense behaviour of the snail is therefore tempting. *Conidae* are known to feed on worms, other gastropods or even fish, interestingly peptides, as for example NPY, have been identified to act as extracellular neuronal messengers in these organisms (Cottrell, 1993; Larhammar et al., 1993). The polypeptide "ANPY toxin" from

Conus anemone could exert its action by trapping messenger molecules of the pancreatic polypeptide family (or related peptides), thereby disturbing the physiological functions of the prey. In support of this hypothesis, it is shown in Fig. 6 that the venom of *Conus anemone* produced a concentration-dependent decrease of the [^{3}H]NPY binding to its receptors in rat cortex membranes under conditions wherein binding of radioligand to the toxin is not measured. Since this action interferes with the function of NPY in the circulatory system (i.e. vasoconstriction) of the animals on which cone snails prey, the ensuing vasodilatation could result in a faster distribution of other, more lethal conotoxins in their organism.

Most of the bioactive components from *Conus* venoms have been shown to be peptides. These "conopeptides" were shown to interact with a variety of cellular targets, especially with voltage controlled and ligand gated ion channels: α-conotoxins bind to nicotinic acetylcholine receptors, μ-conotoxins target sodium channels in muscle, δ-conotoxins bind to molluscan sodium channels, conantokins interact with the glutamate NMDA-receptor and ω-conotoxins block presynaptic calcium channels (Fainzilber et al., 1995; Olivera et al., 1991; Shon et al., 1994). Conotoxins have also been reported to interact with several species of monoamine receptors which belong to the superfamily of G-protein- coupled receptors and they have been used to discriminate between α_2-adrenergic receptors and 5-HT_{1A} serotonergic receptors in the human CNS and between M1- and M2- muscarininc receptor subtypes in calf retina (Czerwiec et al., 1989; Czerwiec et al., 1993; de Vos et al., 1991). The ability of such venom components to interact with receptors for large peptides such as NPY and with such peptides themselves constitute novel additions to the above list of conotoxin actions.

Although *Conus* toxins are well known to deregulate physiological processes by interacting with membrane-bound targets, the present findings suggest that *Conidae* might have developed an additional strategy, involving selective sequestration of peptide messengers in the prey organisms. The present work has been confined to NPY, its analogs and related peptides, but it is evident that such strategy could involve a much wider range of peptide messengers. Since peptides are known to act as neurotransmitters in coelentherates (the first group with recognizable neurones) (Grimmelikhuijzen et al., 1987), components interacting with peptide transmission systems may have evolved in the venom of animals preying on lower invertebrates.

ACKNOWLEDGEMENTS

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