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High-affinity binding of [^{3}H]neuropeptide Y to a polypeptide from the venom of *Conus anemone*

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Abstract

Venom preparation from *Conus anemone* contains a component that binds radiolabeled neuropeptide Y ([^{3}H]neuropeptide Y) with high affinity (K_D = 2.9 nM ± 0.2 nM, B_{max} = 15.2 ± 0.5 pmol/mg protein). Binding of [^{3}H]neuropeptide Y to the venom component is displaced with nanomolar affinity of unlabeled human and porcine neuropeptide Y, porcine [Leu^{31}-Pro^{34}]neuropeptide Y, peptide YY, avian and bovine pancreatic polypeptide, and the (18–36) and (25–36) C-terminal fragments from neuropeptide Y. No displacement is found with the (1–24) N-terminal neuropeptide Y fragment, human secretin, porcine dynorphin A and Boc-DAKLI (Bolton Hunter coupled dynorphin A analog kappa ligand) nor with the non-peptide neuropeptide Y receptor antagonist BIBP3266. Gel filtration chromatography and denaturing (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) show that the [^{3}H]neuropeptide Y-binding component is very likely a single-chain polypeptide with a molecular mass of 18.5 kDa.

Keywords: Neuropeptide Y (NPY); (*Conus* anemone); Binding protein; Pancreatic polypeptide; Neuropeptide Y-(18-36)

1. Introduction

Members of the neogastropod family Conidae (genus *Conus*, ~ 300–500 species) are predators that use potent venoms to paralyze their prey (Kohn, 1959). These venoms are complex mixtures of peptides and polypeptides that interact with a variety of physiological targets. The majority of the investigated conotoxins block neuromuscular and neuronal transmission by interacting with pre- and post-synaptically located ion channels or ligand-gated ion channels (Gray et al., 1988; Olivera et al., 1985, 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 pre-synaptically 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 specicifity which makes them useful as selective pharmacological tools (Cruz et al., 1985; Gray et al., 1988; Groebe et al., 1995) and therapeutic agents (Xiao and Bennet, 1995). Some larger conotoxins have been isolated as well, and were shown to interact with voltage-operated Na^+ channels (striatoxin (25 kDa) from *C. striatus*) and to control the Ca^{2+} homeostasis (eburnetoxin (28 kDa) from *C. eburneus*; tessulatoxin (55 kDa) from *C. tessulatus* and two polypeptide toxins from *C. distans* (24 and 25.5 kDa)) (Kobayashi et al., 1982a,b; Kobayashi et al., 1983; Partoens et al., 1996; Schweitz et al., 1986).

A limited number of conotoxins have, so far, 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). Screening studies revealed that the venoms of certains *Conus* species contain large peptides that prevent the binding of radioligands to their receptors and to related receptors and receptor subtypes (Czerwiec et al., 1989). In this context, it was shown that the venom of *C. tessulatus* is able to discriminate between the M1- and M2-muscarinic receptors as well as between 5-HT_{1A} recep-

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tors and α_2-adrenergic receptors (Czerwiec et al., 1993; De Vos et al., 1991). The latter feature made it possible to selectively study the behavior of [^{3}H]rauwolscine at both receptor types. Screening studies, involving the binding of radiolabeled neuropeptide Y ([^{3}H]neuropeptide Y) (*N*-[propionyl-^{3}H] neuropeptide Y) to its receptor sites in calf brain shed light on a hitherto unexpected property of cone snail venoms (Czerwiec et al., 1996). Instead of inhibiting the binding of the radioligand (a property that could be attributed to the occupancy of the receptors) the venom of *C. anemone* was able to increase [^{3}H]neuropeptide binding in a concentration-dependent way. In the present report, we show that this increase is not related to the presence of receptors and that it could involve binding of [^{3}H]neuropeptide Y to a component in the venom itself. The further characterization and purification of the involved peptide component of *C. anemone* venom is presented.

2. Materials and methods

2.1. Materials

N-[propionyl-^{3}H]neuropeptide Y ([^{3}H]neuropeptide Y) (80 Ci/mmol) was obtained from Amersham (Little Chalfont, UK); neuropeptide Y (porcine), peptide Y (porcine), [Leu^{31}, Pro^{34}]neuropeptide Y (porcine) and neuropeptide Y-(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 (Fraction V) were from Sigma (St. Louis, MO, USA). BIBP3226 was from Albany Molecular Research (Albany, NY, 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.

Biogel P-10 was obtained from Bio-Rad (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).

2.2. Conus anemone venom preparation

Specimens of *C. anemone* were obtained from southwestern 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 0–4°C. The venom ducts were dissected out of the animals, the venom was squeezed out and homogenized in 10 vols. 30 mM ammonium acetate (pH 7.4) (w/v) with a Polytron mixer. Venom homogenate was sonicated 3 times for 10 s in a Soniprep 150 sonicator, centrifugated at 9000 × *g* for 10 min and the resulting supernatants stored at −20°C.

2.3. Rat cortex membrane preparations

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 (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) (pH 7.4)). The homogenate was centrifugated at 30 000 × *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 (30 000 × *g*, 20 min) prior to use.

2.4. Protein concentration determination

Protein concentrations were determined using a modification of the Sopachem ultra-sensitive total protein assay, based on the Pyragallol red-molybdate complex method (Watanabe et al., 1986), with bovine serum albumin as a standard.

2.5. Purification of [^{3}H]neuropeptide Y-binding protein from C. anemone venom by gel filtration chromatography

Venom was prepared as described above and fractionated over a Biogel P10 column (1.5 × 125 cm, elution buffer 50 mM ammonium acetate (pH 7.4)). In a typical experiment, 200 μg of protein were loaded on the column and a pressure head of 13 cm/h was applied. The presence of protein was monitored by absorbance measurement 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 original fraction volume), and assayed for [^{3}H]neuropeptide Y-binding activity as described below.

Fractions containing [^{3}H]neuropeptide Y-binding activity were pooled and an aliquot was taken for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

2.6. SDS-PAGE

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 with PhastGel gradient media'. Staining solutions, and running and staining conditions were as described in the technical files provided by the company.

2.7. Radioligand-binding experiments

C. anemone venom preparation (at the indicated protein concentration), fractions from the Biogel P10 column and/or brain membranes (typically 100 μg protein/assay) were incubated for 60 min at 30°C with [^{3}H]neuropeptide Y. 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. The radioligand concentration ranged between 0.1 and 5 nM for saturation binding experiments and was 0.5 nM for competition binding experiments. Competitor concentrations ranged between 0.01 nM and 1 μM. Fractions of the Biogel P10 column were incubated with 0.5 nM [^{3}H]neuropeptide Y for 60 min at 30°C

After incubation, the samples were rapidly filtered through glass fiber filters (Whatman GF/C, pre-wetted in 0.3% (v/v) polyethyleneimine aqueous solution for 15 min prior to filtration) using a Skatron cell harvester. Filters were washed 4 times with ice-cold buffer (twice 2 s followed by twice 1 s), dried for 10 s in the harvester and in an oven for 60 min at 40°C. Filters were then sealed together with MeltiLex in a sample bag, and radioactivity was counted in a Betaplate.

In centrifugation experiments, *C. anemone* venom preparation (at the indicated protein concentration), rat cortex membrane suspension (typically 100 μg protein/assay) and [^{3}H]neuropeptide Y (0.5 nM) were incubated in a final assay volume of 500 μl, using the same conditions as for filtration experiments. After incubation, mixtures were centrifugated for 10 min at 30 000 × 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. To these 4 ml of Optiphase 'HiSafe', 2 from Wallac (Milton keynes, UK) was added and radioactivity was counted in a liquid scintillation counter.

2.8. Data analysis

Non-specific binding of [^{3}H]neuropeptide Y (0.5 nM) to *C. anemone* venom (10 μg protein/ml) was assessed in the presence of 0.1 μM neuropeptide Y and approximated 20% of the total binding. 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 mean ± S.E.M.

3. Results

3.1. Characterization of the [^{3}H]neuropeptide Y binding to C. anemone venom

Incubation of crude *C. anemone* venom preparation with 0.5 nM [^{3}H]neuropeptide Y for 1 h at 30°C and subsequent vacuum filtration of the mixture over polyethyleneimine (0.3%, v/v)-pre-treated glass fiber filters resulted in the retention of a substantial amount of the radioligand by the filters (Table 1). About 80% of this binding could be displaced by the addition of 0.1 μM unlabeled neuropeptide Y, and since it was not observed in the absence of venom it was defined as specific binding of [^{3}H]neuropeptide Y to one or more of the venom's components. This specific binding could not be detected when the filters had not been pre-treated with polyethyleneimine (Table 1).

Saturation binding experiments, wherein a constant concentration (10 μg protein/ml) of venom was incubated with increasing concentrations of [^{3}H]neuropeptide Y showed that the specific binding occurs with high affinity and is saturable (Fig. 1). Scatchard's plots (Fig. 1, insert) are linear indicating the presence of a homogeneous population of binding sites. The saturation binding parameters ($K_d = 2.95 \pm 0.20$ nM and $B_{max} = 15.2 \pm 0.5$ pmol/mg protein) were determined by non-linear regression analysis. In contrast, the non-specific binding (determined in the presence of 0.1 μM unlabeled neuropeptide Y) increased proportionally to the [^{3}H]neuropeptide Y concentration.

Table 1
Binding of [^{3}H]neuropeptide Y to a component in *C. anemone* venom

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

[^{3}H]Neuropeptide Y (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 pre-treated or pre-treated with (0.3%, v/v) polyethyleneimine. Non-specific binding was measured in the presence of 0.1 μM unlabeled neuropeptide Y. Values refer to radioactivity (cpm) retained on the filter and are means and S.E.M. of 3 experiments. N.D., not determined.

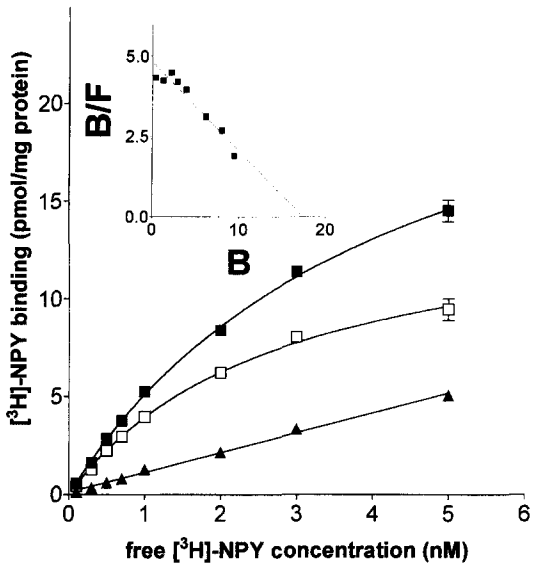

Fig. 1. Saturation binding of [^{3}H]neuropeptide Y to *C. anemone* venom. Venom (10 μg protein/mol) was incubated for 60 min at 30°C with increasing concentrations of [^{3}H]neuropeptide Y (0.1–5 nM) and vacuum-filtered over polyethyleneimine (0.3% v/v) pre-treated Whatmann GF/C filters. Non-specific binding (▲) was measured in the presence of 0.1 μM unlabeled neuropeptide Y. Specific binding (□) was obtained by subtracting non-specific binding from total binding (■). The curves represent means of 3 experiments. The K_D and B_{max} value from the [^{3}H]neuropeptide Y binding is given in Section 3. Insert: Scatchard's plot of the saturation binding data. Curves are means from 3 experiments.

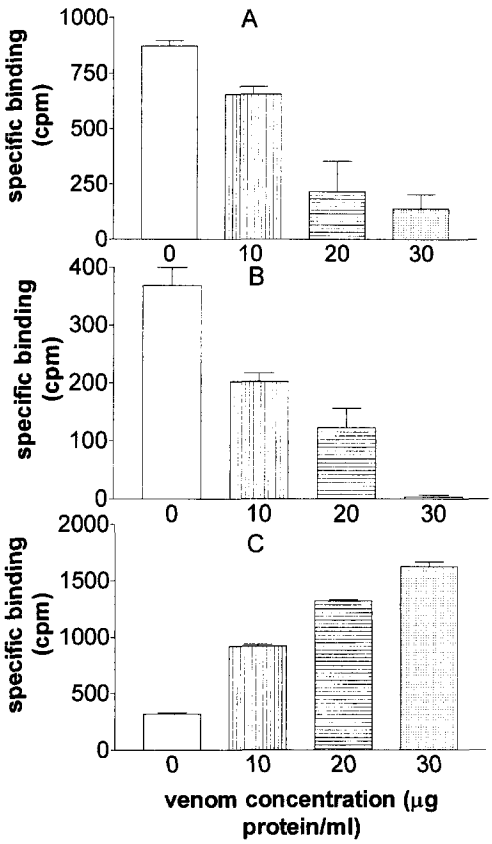

Fig. 2. Effect of *C. anemone* venom on [^{3}H]neuropeptide Y binding to rat cortex membranes. Rat cortex membranes were incubated with 0.5 nM [^{3}H]neuropeptide Y for 60 min at 30°C, in the absence or presence of the indicated concentrations of venom. Mixtures were then subjected to centrifugation (A) and radioactivity of the pellet measured, filtered over non-pre-treated Whatman GF/C filters (B) or filters pre-treated with 0.3% (v/v) polyethylene imine (C). Non-specific binding was measured in the presence of 0.1 μM unlabeled neuropeptide Y. Bars represent means of 3 experiments and error bars represent S.E.M. values.

Specific [^{3}H]neuropeptide Y binding to *C. anemone* venom could be completely displaced with nanomolar affinity by unlabeled human and porcine neuropeptide Y, porcine [Leu^{31}, Pro^{34}]neuropeptide Y, porcine peptide YY and avian and bovine pancreatic polypeptide (Table 2). 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 neuropeptide Y but not with the (1–24) N-terminal fragment

Table 2
Competition binding parameters of various peptides for the specific [^{3}H]neuropeptide Y binding to *C. anemone* venom

Peptide	Amino-acid sequence	IC_{50} (nM)
Human neuropeptide Y	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY	8.7 ± 1.0
Porcine neuropeptide Y	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY	9.3 ± 1.0
Human neuropeptide Y-(1-24)	YPSKPDNPGEDAPAEDMARYYSAL—	> 1000
Neuropeptide Y-(18-36)	— ARYYSALRHYINLITRQRY	29.5 ± 1.0
Neuropeptide Y-(25-36)	—RHYINLITRQRY	5.5 ± 0.9
Porcine [Leu^{31}, Pro^{34}]neuropeptide Y	YPSKPDNPGEDAPAEDLARYYSALRHYINLLTRPRY	2.6 ± 1.0
Porcine peptide YY	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY	7.4 ± 1.1
Avian pancreatic polypeptide	GPSQPTYPGDDAPVEDLIRFYDNLQQYLNVVTRHRY	1.5 ± 0.15
Bovine pancreatic polypeptide	APLEPEYPGDDATPEQMAQYAAELRRYINMLTRPRY	2.3 ± 0.9
Human secretin	HSDGTFTSELSRLREGARLQRLLQGLV	> 1000
Porcine dynorphin A	YGGFLRRIRPKLKWDNQ	> 1000
Boc-DAKLI	*N-t*-Boc-YGGFLRRIRPRLRG-5-aminopentylamide	> 1000

Amino-acid sequences of tested peptides is given together with the respective IC_{50} values. Mixtures of [^{3}H]neuropeptide Y (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) pre-treated 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 3 experiments.

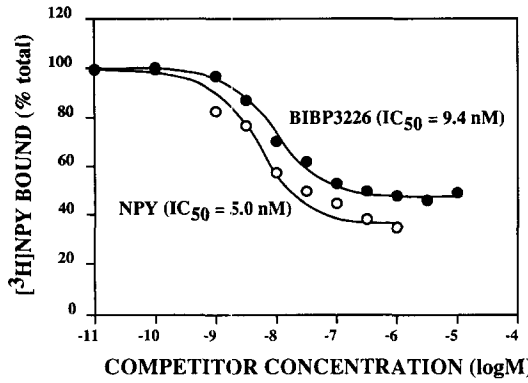

Fig. 3. Competition binding on neuropeptide Y receptors from rat cortex membranes. Membranes were incubated with [^{3}H]neuropeptide Y (0.5 nM) and increasing concentrations of unlabeled neuropeptide Y (○) and the non-peptide selective neuropeptide Y Y_1 receptor antagonist BIBP3226 (●). Data were obtained in 1 representative experiment with each point determined in triplicate.

of human neuropeptide Y. 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 2), nor with the

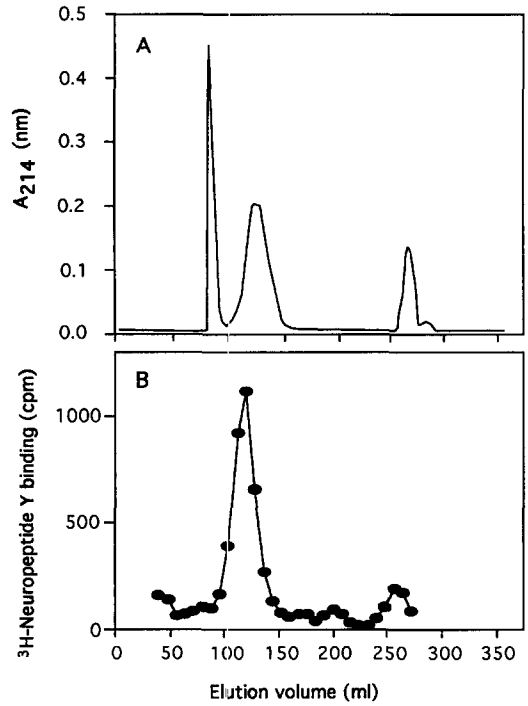

Fig. 4. (A) Gelfiltration chromatogram of *C. anemone* venom fractionation experiment. A Biogel P10 column (1.5×125 cm, elution buffer 50 mM ammonium acetate (pH 7.5)) was loaded with 200 μg of protein dissolved in elution buffer. Fractions of 10 ml were collected and lyophilized, resuspended in elution buffer (10% of the original fraction volume) and tested for [^{3}H]neuropeptide Y-binding activity as described in Section 2. Three major peaks are detected by absorbance measurement at 214 nm. (B) Specific binding of [^{3}H]neuropeptide Y (0.5 nM) above baseline values (in the absence of fractionated material). Other fractionation experiments gave identical results.

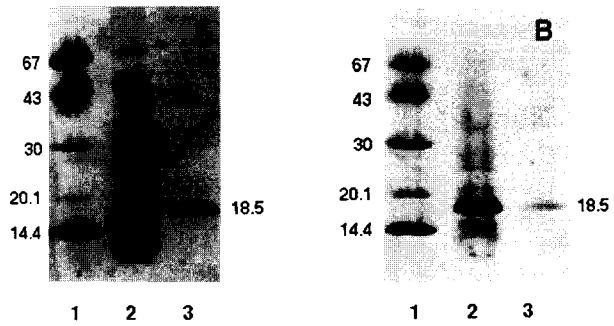

Fig. 5. SDS-PAGE of crude *C. anemone* venom and [^{3}H]neuropeptide Y-binding protein. Standard protein mixture (lane 1, A and B), crude venom preparation (lane 2, A and B) and [^{3}H]neuropeptide Y-binding protein (lane 3, A and B) were loaded on gradient gels (8–25%). Identical samples (typically 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. 4.

non-peptide neuropeptide Y receptor antagonist BIBP3226 (data not shown).

A substantial proportion of the [^{3}H]neuropeptide Y binding to rat cortex membranes could be displaced with unlabeled neuropeptide Y (IC_{50} = 5 nM) and about 80% of this specific (i.e., neuropeptide Y displaceable) binding displayed high affinity for the neuropeptide Y Y_1-selective antagonist BIBP3226 (IC_{50} = 6.3 nM, Fig. 3). A similar predominance of neuropeptide Y Y_1 receptors has also been reported by others by using less selective compounds (Dumont et al., 1993). When added to this mixture, the venom of *C. anemone* produced a concentration-dependent increase in specific binding of [^{3}H]neuropeptide Y when free and bound radioligand was separated by filtration on polyethyleneimine-treated filters (Fig. 2CFig. 3). This increase may be attributed to the trapping of venom-bound [^{3}H]neuropeptide Y by the filters. Specific binding of [^{3}H]neuropeptide Y to its receptors still occurred when separations were performed by using non-pre-treated filters or by centrifugation but, under these conditions, the venom of *C. anemone* produced a concentration-dependent decrease in binding (Fig. 2A,B).

3.2. Purification of the [^{3}H]neuropeptide Y-binding protein from C. anemone venom

SDS-PAGE from crude *C. anemone* extract reveals that some high-molecular-mass components are present and that a fair amount of low-molecular-mass components is detected by silver staining (Fig. 5A). The major component of the venom has a molecular mass of 18.5 kDa (Fig. 5). During fractionation of the crude *C. anemone* venom over a Biogel P10 gel-filtration resin (fractionation range 1.5–20 kDa), three major absorbance peaks are detected (at 214 nm (Fig. 4A) and a1.5–2t 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 molecular mass > 20 kDa. The [^{3}H]neuropeptide Y-binding activity was confined to the second peak and was insignificant in any of the other fractions (Fig. 4B). SDS-PAGE of material from the second peak shows the presence 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. 5A,B).

4. Discussion

Earlier experiments, wherein crude *Conus* venoms were screened for their ability to interact with neuropeptide Y receptors in calf brain revealed, rather unexpectedly, the ability of *C. anemone* venom to increase the binding of the radioligand [^{3}H]neuropeptide Y (Czerwiec et al., 1996). Control experiments showed that this binding was unrelated to the presence of the receptors or membranes, suggesting that one or more components of the venom were involved instead. The present report deals with the isolation of a [^{3}H]neuropeptide Y-binding peptide component of the *C. anemone* venom, further denominated as 'ANPY' toxin, and with the structural requirements for the binding of neuropeptide Y and some related peptides to take place.

SDS-PAGE of crude *C. anemone* venom shows that it contains several polypeptide components with high molecular mass and that one of them (18.5 kDa) is particularly abundant. When the crude venom was subjected to gel-filtration chromatography over a column with a fractionation range of 1.5–20 kDa, the [^{3}H]neuropeptide Y-binding activity was found to elute in a single peak, immediately after the void volume (Fig. 4B). SDS-PAGE of the active fraction revealed a substantial enrichment of the 18.5-kDa polypeptide (Fig. 5). It is thus very likely that the ANPY toxin corresponds to this polypeptide. However, conclusive proof awaits further experiments, such as [^{3}H]neuropeptide Y binding to a purified preparation and/or cross-linking. The ANPY toxin displays high affinity for neuropeptide Y (K_D of 3 nM by saturation binding, and IC_{50} of 8.7 nM by competition binding; Fig. 1, Table 2) as well as for other relatives of the pancreatic polypeptide family, such as peptide YY and avian and bovine pancreatic polypeptide (Table 2).

Competition binding experiments with neuropeptide Y fragments reveal that the N-terminal fragment neuropeptide Y-(1–24) is devoid of binding activity while the C-terminal terminal fragments neuropeptide Y-(18–36) and neuropeptide Y-(25–36) are nearly as active as the native peptide. These data clearly show that the C-terminal portion of neuropeptide Y is crucial for the binding to the toxin. Along the same line, it has been shown that C-terminal fragments of neuropeptide Y 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 neuropeptide Y-(25–36), display already over 10 000-times lower affinity for the Y_2 receptors in rat hippocampus as compared to neuropeptide Y (Danho et al., 1988). Accordingly, it is also interesting to notice that the affinity of neuropeptide Y-(25–36) for the Y_2 receptors is about 500 times lower than that for the toxin. This distinction suggests that the binding epitope on the toxin is quite different from those on the neuropeptide Y receptors.

When the filtration technique is used to measure the binding of [^{3}H]neuropeptide Y to its receptors in membrane preparations, the glass fiber filters are routinely pre-treated with positively charged polyethyleneimine to reduce the extend of non-specific binding of the radioligand to the filters. This pre-treatment appears to be essential for retaining the [^{3}H]neuropeptide Y toxin complexes (Table 1) and it is therefore likely that ionic interactions between the positively charged filters and a negatively charged moiety of the toxin are involved.

Spatial aspects of neuropeptide Y 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 neuropeptide Y 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 spatial segregation of the positively and negatively charged groups on the neuropeptide Y molecule, it is plausible that neuropeptide Y (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 neuropeptide Y as compared to the very weak affinity of the negatively charged (1–24) N-terminal fragment. Neuropeptide Y-(25–36), the shortest of the active neuropeptide Y fragments tested, contains three Arg 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 neuropeptide Y molecule. In this context, neuropeptide Y 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 neuropeptide Y in this process has clearly been evidenced (Fuhlendorff et al., 1990; Mousli and Landry, 1994; Mousli et al., 1994, 1995). However, since very little is known about the molecular mechanisms which are involved in guiding the neuropeptide Y molecule to its intracellular target, the G-protein, it is unclear whether the

positive charge of neuropeptide Y (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., 1994).

Yet, a model involving merely electrostatic interactions between the positively charged N-terminal epitope of neuropeptide Y and a negatively charged moiety on the toxin appears to be too simple to explain the neuropeptide Y–toxin interaction. Indeed, it does not explain why peptides of the pancreatic polypeptide family with an Arg residue at position 25 (neuropeptide Y, peptide YY), Arg 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 2). These peptides have Arg 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]neuropeptide Y binding from the toxin (Table 2). The active neuropeptide Y-related peptides all have a C-terminal Tyr residue, but the Arg-Tyr sequence is not sufficient for binding to the toxin either, since BIBP3226, a neuropeptide Y 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 neuropeptide Y 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 neuropeptide Y-(27–36), as well as of modified fragments might provide more precise information about the nature and location of the amino acids of neuropeptide Y which are involved in the binding to the 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 an association with preying or defense behavior of the snail is therefore tempting. Conidae are known to feed on worms, other gastropods or even fish. Interestingly, peptides, e.g., neuropeptide Y, have been identified to act as extracellular neuronal messengers in these organisms (Cottrell, 1993; Larhammar et al., 1993). The polypeptide 'ANPY toxin' from *C. anemone* could eventually 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. 2 that the venom of *C. anemone* produced a concentration-dependent decrease in the [^{3}H]neuropeptide Y binding to its receptors in rat cortex membranes under conditions wherein binding of radioligand to the toxin is not measured (i.e., when binding to the receptors was measured either by filtration on filters which were not pre-treated with polyethyleneimine or by centrifugation).

The majority of the *Conus* toxins characterized to date exert their action by binding to membrane-bound targets, thereby impairing or completely blocking neuronal and neuromuscular transmission. Although this is likely to be the preferred and maybe most effective solution to deregulate physiological processes, 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 neuropeptide Y, 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.

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References

Allen, J., J. Novotny, J. Martin and G. Heinrich, 1987, Molecular structure of mammalian neuropeptide Y: analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue, Proc. Natl. Acad. Sci. USA 84, 2532.

Boublik, J., N. Scott, J. Taulane, M. Goodman, M. Brown and J. Rivier, 1989, Neuropeptide Y and neuropeptide Y_{18-36}, Int. J. Pept. Protein Res. 33, 11.

Cottrell, G.A., 1993, The wide range of actions of the FMRF amide-related peptides and the biological importance of peptidergic messengers, in: Comparative Molecular Neurobiology, ed. Y. Pichon (Birkhauser, Basel) p. 278.

Cruz, L.J., V. De Santos, G.C. Zafaralla, C.A. Ramilo, R. Zeikus, W.R. Gray and B.M. Olivera, 1987, Invertebrate vasopressin/oxytocin homologs, J. Biol. Chem. 262, 15821.

Cruz, L.J., W.R. Gray, B.M. Olivera, R.D. Zeikus, L. Kerr, D. Yoshikami and E. Moczydlowski, 1985, *Conus geographus* toxins that discriminate between neuronal and sodium channels, J. Biol. Chem. 260, 9280.

Czerwiec, E., J.-P. De Backer, W. De Potter and G. Vauquelin, 1993, Selective masking of M1-receptors in calf retina membranes by the venom of the marine snail *Conus tessulatus*, Neurochem. Int. 23, 79.

Czerwiec, E., J.-P. De Backer, G. Vauquelin and P. Vanderheyden, 1996, Neuropeptide Y receptors from calf brain: effect of crude *Conus* venom preparations on [^{3}H]neuropeptide Y binding, Neurochem. Int. (in press).

Czerwiec, E., W. De Potter, A. Convents and G. Vauquelin, 1989, *Conus* venom interaction with α_2-adrenergic receptors in calf retina membranes, Neurochem. Int. 14, 413.

Danho, W., J. Triscari, G. Vincent, T. Nakajima, J. Taylor and E.T. Kaiser, 1988, Synthesis and biological evaluation of pNeuropeptide Y fragments, Int. J. Pept. Protein Res. 32, 496.

De Vos, H., E. Czerwiec, J.-P. De Backer, W. De Potter and G. Vauquelin, 1991, [^{3}H]Rauwolscine behaves as an agonist for the 5-HT_{1A} receptors in human frontal cortex membranes, Eur. J. Pharmacol. 207, 1.

Dumont, Y., A. Fournier, S. St.-Pierre and R. Quirion, 1993, Comparative characterization and autoradiographic distribution of neuropeptide Y receptor subtypes in the rat brain, J. Neurosci. 13, 73.

Fox, A.P., K.D. Hirning, S. Kongsamut, E.W. McCleskey, R.J. Miller, B.M. Olivera, T.M. Perney, S.A. Thayer and R.W. Tsien, 1987, The interactions of toxins with calcium channels, in: Neurotoxins and Their Pharmacological Implications, ed. P. Jenner (Raven Press, New York, NY) p. 115.

Fuhlendorff, J., N. Langeland Johansen, S.G. Melberg, H. Thøgersen and T.W. Schwartz, 1990, The antiparallel pancreatic polypeptide fold in the binding of neuropeptide Y to Y_1 and Y_2 receptors, J. Biol. Chem. 265, 11706.

Glover, J.D., D.J. Barlow, J.E. Pitts, S.P. Wood, I.J. Tickle, T.L. Blundell, K. Tatemoto, J.R. Kimmel, A. Wollmer, W. Strassburger and Y.S. Zhang, 1985, Conformational studies of the pancreatic polypeptide hormone family, Eur. J. Biochem. 142, 379.

Gray, W.R., B.M. Olivera and L.J. Cruz, 1988, Peptide toxins from venomous *Conus* snails, Annu. Rev. Biochem. 57, 665.

Grimmelikhuijzen, C.J.P., A. Groeger and I.D. Mcfarland, 1987, Neuropeptides in invertebrates, in: Nervous Systems in Invertebrates, ed. M.A. Ali (Plenum Press, New York, NY) p. 105.

Groebe, D.R., J.M. Dumm, E.S. Levitan and S.N. Abramson, 1995, α-Conotoxins selectively inhibit one of the two acetylcholine binding sites of nicotinic receptors, Mol. Pharmacol. 48, 105.

Jung, G., A.G. Beck-Sickinger, H. Dürr, W. Gaida and G. Schnorrenberg, 1991, α-Helical small molecular size analogues of neuropeptide Y: structure-activity relationships, Biopolymers 31, 613.

Kobayashi, J., H. Nakamura, Y. Hirata and Y. Ohizumi, 1982a, Isolation of a cardiotonic glycoprotein, striatoxin, from the venom of the marine snail *Conus striatus*, Biochem. Biophys. Res. Commun. 105, 1389.

Kobayashi, J., H. Nakamura, Y. Hirata and Y. Ohizumi, 1982b, Isolation of eburnetoxin, a vasoactive substance from the *Conus eburneus* venom, Life Sci. 31, 1085.

Kobayashi, J., H. Nakamura, Y. Hirata and Y. Ohizumi, 1983, Tessulatoxin, the vasoactive protein from the venom of the marine snail *Conus tessulatus*, Comp. Biochem. Physiol. B 74, 381.

Kohn, A.J., 1959, The ecology of *Conus* in Hawaii, Ecol. Monogr. 29, 47.

Larhammar, D., C. Söderberg, A. G. Blomqvist, 1993, Evolution of the neuropeptide Y family of peptides, in: The Biology of Neuropeptide Y and Related Peptides, ed. W.F. Colmers and C. Wahlestedt (Humana Press, Totowa, NJ) p. 1.

McKerell, A.D.J., 1988, Molecular modelling and dynamics of neuropeptide Y, J. Comput.-Aided Mol. Design 2, 55.

Minakata, H., J.W. Taylor, M.W. Walker, R.J. Miller and E.T. Kaiser, 1989, Characterization of amphiphilic secondary structures in neuropeptide Y through the design, synthesis and study of model peptides, J. Biol. Chem. 264, 7907.

Mousli, M., T.E. Hugli, Y. Landry and C. Bronner, 1994, Peptidergic pathway in human skin and rat peritoneal mast cell activation, Immunopharmacology 27, 1.

Mousli, M. and Y. Landry, 1994, Role of positive charges of neuropeptide Y fragments in mast cell activation, Agents Act. 41, C41.

Mousli, M., A. Trifilieff, J.T. Pelton, J.-P. Gies and Y. Landry, 1995, Structural requirements for neuropeptide Y in mast cells and G protein activation, Eur. J. Pharmacol. 289, 125.

Olivera, B.M., W.R. Gray, R. Zeikus, J.M. McIntosh, J. Varga, J. Rivier, V. De Santos and L.J. Cruz, 1985, Peptide neurotoxins from fish-hunting cone snails, Science 230, 1338.

Olivera, B.M., G.P. Miljanich, J. Ramachandran and M.E. Adams, 1994, Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins, Annu. Rev. Biochem. 63, 823.

Olivera, B.M., J. Rivier, C. Clark, C. A. Ramilo, G.P. Corpuz, F.C. Abogadie, E.E. Mena, S.R. Woodward, D.R. Hillyard and L.J. Cruz, 1990, Diversity of *Conus* neuropeptides, Science 249, 257.

Olivera, B.M., J. Rivier, J.K. Scott, D. R. Hillyard and L.J. Cruz, 1991, Conotoxins, J. Biol. Chem. 266, 22067.

Partoens, P., J.M. Wang, E.P. Coen, G. Vauquelin and W.P. De Potter, 1996, Two polypeptide toxins with opposite effects on calcium uptake in bovine chromaffin cells: isolation from the venom of the marine snail *Conus distans*, Neurochem. Int. (in press).

Rudolf, K., W. Eberlein, W. Engel, H. A. Wieland, K.D. Willim, M. Entzeroth, W. Wienen, A.G. Beck-Sickingern and H.N. Doods, 1994, The first highly potent and selective non-peptide neuropeptide Y Y_1 receptor antagonist: BIBP3226, Eur. J. Pharmacol. 271, R11.

Schwartz, T.W., J. Fuhlendorff, L.L. Kjems, M.S. Kristensen, M. Vervelde, M. O'Hare, J.L. Kretenansky and B. Björnholm, 1990, Signal epitopes in the three-dimensional structure of Neuropeptide Y interaction with Y_1, Y_2 and PP receptors, Ann. N.Y. Acad. Sci. 611, 35.

Schweitz, H., J.-F. Renaud, N. Randimbivololona, C. Préau, A. Schmid, G. Romey, L.H. Rakotovao and M. Lazdunski, 1986, Purification, subunit structure and pharmacological effects on cardiac and smooth muscle cells of a polypeptide toxin isolated from the marine snail *Conus tessulatus*, Eur. J. Biochem. 161, 787.

Sheikh, S.P., R. Hakanson and T.W. Schwartz, 1989, Y_1 and Y_2 receptors for neuropeptide Y, FEBS Lett. 245, 209.

Watanabe, N., S. Kamei, A. Ohkubu, M. Yamanaka, S. Ohsawa, K. Makino and K. Tokuba, 1986, Urinary protein as measured with a pyrogallol red-molybdate complex, manually and in Hitachi 726 automated analyser, Clin. Chem. 32, 1551.

Xiao, W.-H. and G.J. Bennet, 1995, Synthetic ω-conopeptides applied to the site of nerve injury suppress neuropathic pains in rats, J. Pharmacol. Exp. Ther. 274, 666.