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High Affinity Displacement of [^{3}H]NPY Binding to the Crude Venom of *Conus anemone* by Insect Neuropeptides

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The venom from *Conus anemone* contains a protein, named ANPY toxin, which displayed high affinity (IC_{50} in nanomolar range) to neuropeptide Y (NPY), [Leu^{31}, Pro^{34}]NPY, peptide YY, pancreatic polypeptide, the Y_1 antagonist 1229U91, and C-terminal NPY fragments. N-terminal fragments and the free acid form of NPY did not bind to ANPY. The truncated NPY fragments displayed very low affinity to Y_1 receptors and partially inhibited [^{3}H]NPY binding to anti-NPY antiserum. Several insect neuropeptides, the sequences of which related to the C-terminal fragments of NPY, were observed to bind with similar affinity or even 20 times higher (Lom-MS and Scg-NPF) affinity than NPY. In contrast, no significant binding of these insect peptides was observed for Y_1 receptors and anti-NPY antiserum. Therefore, ANPY can be viewed as an acceptor that binds with very high affinity to a broad spectrum of vertebrate and invertebrate neuropeptides that share a similar C-terminal amino acid sequence.

***Key Words: Conus anemone;* insect peptide; NPY; Y_1 receptors; anti-NPY antibodies.**

The tropical marine gastropods *Conus* (Family *Toxoglossa*) are capable of preying and self-defending by producing venomous toxins that quickly immobilize the preys or predators (1). All *Conus* species possess a similar venom apparatus, where the venom is formed in a long tubular duct (venom duct). Different toxins from cone snails have been shown to interact with specific receptors, voltage-gated ion channels and ligand-gated ion channels. The μ-conotoxins, δ-conotoxins block neuronal transmission of voltage-sensitive sodium channels, and ω-conotoxins block voltage-sensitive calcium channels. Other conotoxins have been shown to interact with neurotransmitter receptors referred to ligand-gated ion channels such as nicotinic acetylcholine receptors (α-conotoxins), NMDA-glutamate receptors (conatokins), and serotonin receptors (2–6). Alternatively, certain *Conus* venoms were also reported to interfere with G-protein coupled receptors, such as vasopressin receptors, α_2-adrenergic and muscarinic cholinergic receptors, neurotensin receptors (contulakin-G), and neuropeptide Y receptors (7–12). At present no less than four peptides are in clinical trails for use as analgesics, the control of epilepsy, the diagnosis of auto immune diseases (5, 13–15).

The crude venom of *C. anemone* was found to contain a protein toxin, called ANPY, which binds to neuropeptide Y (NPY) with high affinity (11). The [^{3}H]NPY binding to ANPY was displaced by peptide YY (PYY) or pancreatic polypeptide (PP) at nanomolar concentrations, but not by the NPY Y_1 receptor selective nonpeptide antagonist, BIBP3226. Furthermore, competition binding with the C-terminal fragments of NPY(18-36) and NPY(25-36) suggested that the C-terminal NPY motif might be involved in the binding to ANPY (11).

Neuropeptide Y is a member of the pancreatic polypeptide family. The sequence of NPY is extremely well conserved, underlining it has very important functions and/or multiple points of interaction with target molecules. In many invertebrates, NPY-like peptides contain the ultimate phenylalanine (Phe or F) residue and are therefore designated as NPF. NPF/NPY-like peptides are widely distributed in the central, peripheral and enteric nervous system of species extending from worms to humans (16–19).

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In the present study, we investigated the ANPY binding to NPY fragments and analogs and of NPF-like peptides that were isolated from brain extracts of the Colorado Potato beetle (*Leptinotarsa decemlineata*) (20). The C-terminal part of these insect peptides exhibits sequence similarity with the corresponding C-terminal part of invertebrate NPF and vertebrate NPY. In addition, we have screened a number of arthropod peptides, including myotropins (21, 22) originating from the migratory locust (*Locusta migratoria*) and the desert locust (*Schistocerca gregaria*). The binding of these peptides to ANPY was compared with their ability to displace [^{3}H]NPY binding to CHO-cells expressing human Y_1 receptors and to a commercial antiserum against NPY.

MATERIALS AND METHODS

Materials. *N*-(*propionyl*)[^{3}H]neuropeptide Y was obtained from Amersham (Little Chalfont, UK). Human NPY(1-36), human NPY(1-24), and human and porcine PYY were from PolyPeptide Laboratories GmbH (Germany). Porcine [Leu^{31},Pro^{34}]NPY, porcine NPY(18-36) and bovine pancreatic polypeptide (PP) were from Sigma (USA). [His^{32},Leu^{34}]NPY(32-36), and human NPY free acid (NPY-COOH) were from Bachem (Switzerland). RAS 7172, rabbit anti-(porcine)NPY serum, was from Peninsula Laboratories, INC. NPY receptor antagonist 1229U91 was from Neosystem (Strasbourg, France). Porcine NPY(25-36) was a kind gift from Prof. J. Lundberg (Karolinska Institute, Sweden). Wild type cells (CHO-K1) were kindly donated by Dr. H. Verschueren (Pasteur Institute, Brussels, Belgium) and CHO cells stably expressing the human NPY Y_1 receptor (denoted as CHO-Y_1 cells) (23).

All the arthropod peptides including Aea-TMOF, Corazonin, Lom-AG-MT1, Lom-AG-MT2, Lom-MIP, Lom-MS, Lom-MT1, Lom-MT3, Lom-PK2, Led-NPF1, Led-NPF2, Pev-KIN1, Scg-AST7, Scg-MT1, Scg-MT2 and Scg-NPF were synthesized using Fmoc polyamide chemistry (J. W. Drijfhout, University Hospital of Leiden, The Netherlands). All other chemicals were of reagent grade.

Conus anemone venom preparation. Frozen *Conus anemone* snails were obtained from South-West Australia. The dissection was carried out at 0–4°C using 50 mM ammoniumacetate (pH 7.4) containing 0.1 μM phenylmethyl sulphonyl floride (PMSF). The venom was extracted from the duct apparatus by squeezing and homogenizing with a Polytron mixer. Venom homogenate was sonicated three times for 10 s in a Soniprep 150 sonicator, then centrifuged at 9000 $\times$ g for 15 min. The supernatant was collected, lyophilized, and stored at $-$20°C until use.

Competition binding assay to C anemone venom. Crude *C. anemone* venom was resuspended in Krebs-Ringer buffer (137 mM NaCl/ 2.68 mM KCl/2.05 mM $MgCl_2$/1.8 mM $CaCl_2$/20 mM Hepes) (pH 7.4) at a final protein concentration of 50 mg/ml. Radioactive [^{3}H]NPY and test peptides were diluted in Krebs-Ringer buffer containing 0.2% BSA. All assays were performed in plastic 96-well plates in a 200 μl volume consisting of 100 μl diluted crude venom, 50 μl of [^{3}H]NPY (at a final concentration of 2 nM) and 50 μl buffer or test compound at the final concentrations between 0.1 nM to 10 μM. Incubation was proceeded for 60 min at 30°C. After the incubation, the samples were rapidly filtered through glass fiber filters (Whatman GF/C filter, presoaked in 0.3% polyethylenimine for 5 min) using a Skatron Cell Harvester. Filters were washed four times with ice-cold Krebs-Ringer buffer, dried for 10 s in the harvester and for 60 min at 50°C in an oven. The dried filters were sealed with MeltiLex melt-on scintillation sheets in a sample bag, and the radioactivity was counted using a Wallac 1205 Betaplate counter. Non-specific binding was determined in the presence of 1 μM unlabelled NPY.

Competition binding assay to anti-NPY antiserum. Lyophilized anti-NPY antiserum was rehydrated with 25 ml of 0.1% Triton X-100. 100 μl of this solution was incubated for 1 hour at room temperature with a final concentration of 2 nM [^{3}H]NPY in a total volume of 200 μl buffer containing 19 mM $NaHPO_4$, 81 mM Na_2HPO_4, 50 mM NaCl, and 0.1% BSA. After the incubation, the samples were applied to a Sephadex G-50 gel filtration column (0.7 $\times$ 30 cm) pre-equilibrated with phosphate buffer. The void volume (3 ml) was decanted. The fraction containing anti-NPY-[^{3}H]NPY complexes (3 ml) were eluted and counted in a Liquid Scintillation counter. Finally the fraction containing [^{3}H]NPY (5 ml) was eluted for regeneration of the columns. Non-specific binding was determined in the presence of 1 μM unlabelled NPY. Competition binding was performed by including the unlabelled tested compounds at concentrations from 1 nM to 10 μM.

Competition binding assay to NPY Y_1 receptors. The CHO-K1 cells, expressing human NPY Y_1 receptors were plated in 24-well-plates and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 2 mM L-glutamine, 100 U.I./ml penicillin, 100 μg/ml streptomycin, 1% of a solution containing non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. The cells were grown in 5% CO_2 at 37°C until they reached confluence. Before the binding experiment, the medium was removed and the cells were washed twice with 0.5 ml per well of DMEM. After a preincubation for 30 min at room temperature in 400 μl DMEM containing 0.5% BSA and 0.1 μM PMSF, 50 μl of [^{3}H]NPY (final concentration of 0.5-1 nM) and 50 μl of the tested peptides (at the indicated concentrations) were added and the cells were further incubated for 90 min at 37°C. The incubations were terminated by placing the plates on ice and washing the cells three times with ice-cold Krebs-Ringer buffer. Bound [^{3}H]NPY was determined after solubilization of the cells with 0.5 ml of 0.6 M NaOH for 1 hour at room temperature. The radioactivity was counted in a Liquid Scintillation counter. Non-specific binding was measured in the presence of 1 μM unlabelled NPY.

RESULTS

Screening of NPY analogs and insect peptides. A number of NPY-related peptides at a final concentration of 10 μM were tested for their ability to displace specific binding of [^{3}H]NPY to the crude venom of *C. anemone* (ANPY), NPY Y_1-receptors and an antiserum directed against NPY. Specific binding of [^{3}H]NPY was determined by subtracting the radioligand binding in the presence of 1 μM unlabelled NPY from the total binding. As shown in Figure 1A, specific [^{3}H]NPY binding to ANPY was completely displaced by unlabeled human NPY and analogs, except [His^{32}Leu^{34}]NPY(32-36) and NPY-COOH which only partially displaced [^{3}H]NPY binding. No displacement was seen for NPY(1-24). A similar binding profile was observed for CHO- Y_1 receptors (Figure 1B), except that bovine PP and the C-terminal NPY(25-36) only marginally displaced [^{3}H]NPY binding.

In Figure 1C, specific binding of [^{3}H]NPY to the NPY-antiserum could be completely displaced by NPY itself and by [Leu^{31}Pro^{34}]NPY. No displacement was seen for [His^{32}Leu^{34}]NPY(32-36) and only partial displacement (between 48 to 66%) was seen for all the other analogs.

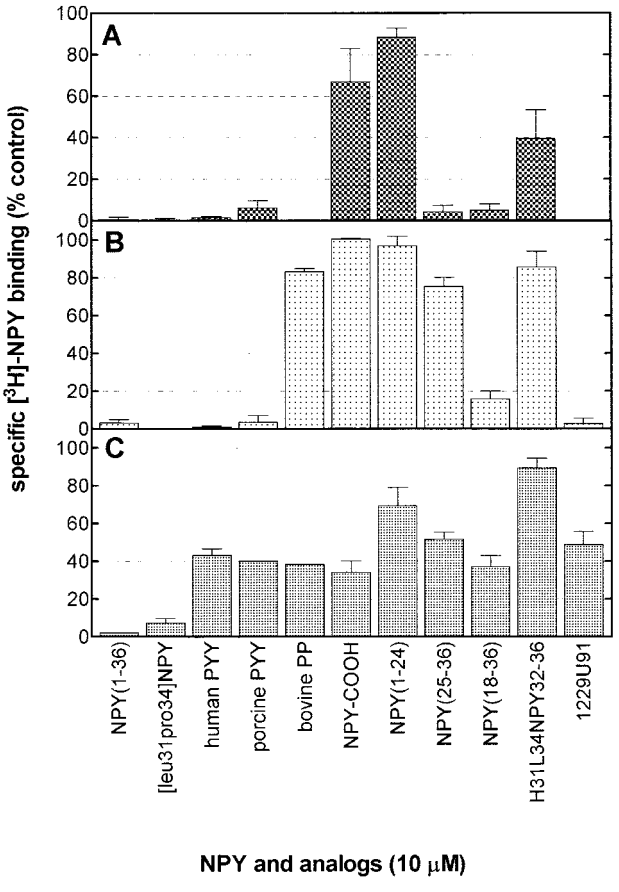

FIG. 1. Displacement of [^{3}H]NPY binding to ANPY toxin (A), Y_1 receptors in CHO-Y_1 cells (B), and anti-NPY antiserum (C) by NPY and analogs. Bars represent the specific [^{3}H]NPY binding (in % of control binding) in the presence of 10 μM peptide.

Subsequently, 16 invertebrate peptides were screened for their binding to ANPY toxin, Y_1 receptors and anti-NPY antiserum in the same conditions as above. Pev-KIN1 is a myokinin-like peptide that was discovered in the white shrimp, *Penaeus vannamei* (24). Aea-TMOF is a trypsin modulating and oostatic peptide produced in the ovaries of the mosquito *Aedes aegypti* (25). Led-NPF1 and Led-NPF2 (20) are NPF-like peptides from Colorado potato beetle (*Leptinotarsa decemlineata*). All other investigated peptides are derived from *Locusta migratoria* and *Schistocerca gregaria* (21, 22, 26). As illustrated in Figure 2A, nine peptides completely inhibited the binding of [^{3}H]NPY to *C. anemone* venom (panel A) at 10 μM. A near maximal displacement was obtained with Scg-AST7 and Lom-AG-MT1. The other tested peptides were inactive. In contrast, there was no significant, measurable binding of these insect peptides to the Y_1 receptors (panel B). Most of the insect peptides showed no cross-reactivity with the anti-NPY antiserum. Nevertheless, a limited displacement of [^{3}H]NPY was observed for a few peptides, especially for Led-NPF1, an insect peptide that displays sequence similarity to the C-terminal part of NPY.

Competition binding experiments. Further comparison of the [^{3}H]NPY displacing peptides was done by competition binding experiments to ANPY, human NPY Y_1 receptors and the NPY antiserum. The obtained IC_{50} values, given in Table 1, were calculated by non-linear regression analysis. NPY, PYY, PP, the C-terminal NPY fragments as well as the peptide NPY Y_1 antagonist 1229U91 displaced [^{3}H]NPY binding from ANPY at nanomolar concentrations. Similar or even higher affinities were observed for the insect peptides Lom-MS, Scg-NPF and Lom-MT3 (Table 1). Competition binding curves with the six other insect peptides that were positive in the screening tests revealed that they interacted with ANPY with higher IC_{50} values.

The potency order for displacement of [^{3}H]NPY from NPY Y_1 receptors by NPY related peptides was NPY $\approx$ [Leu^{31}Pro^{34}]NPY $\approx$ PYY $\approx$ 1229U91 $\gg$ NPY(18-36) $\gg$ PP $\approx$ NPY(25-36) $\approx$ NPY-COOH. None of the tested invertebrate peptides showed significant displacement of [^{3}H]NPY binding to NPY Y_1 receptors at concentrations of 10 μM.

NPY displaced [^{3}H]NPY binding to the antiserum with an IC_{50} value of 17 nM. NPY-COOH, [Leu^{31}Pro^{34}]NPY, PYY, PP, 1229U91 as well as the N- and C-terminal

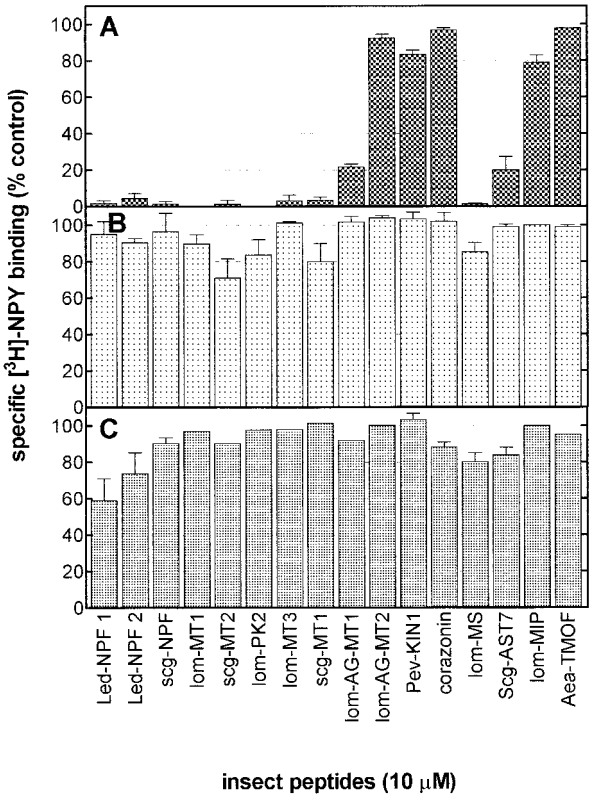

FIG. 2. Displacement of [^{3}H]NPY binding to ANPY toxin (A), Y_1 receptors in CHO-Y_1 cells (B), and anti-NPY antiserum (C) by insect peptide. Bars represent the specific [^{3}H]NPY binding (in % of control binding) in the presence of 10 μM peptide.

TABLE 1

Competition Binding (IC_{50}) of Various Neuropeptides and Insect Peptides on the Specific [^{3}H]NPY Binding to *C. anemone* Venom, Anti-NPY Antiserum, and Y_1 Receptors

Peptides	ANPY toxin[a] IC_{50} (nM)	Y_1 receptors IC_{50} (nM)	NPY antiserum IC_{50} (nM)
NPY and analogs			
h NPY	13	2.9	17
p [L^{31}P^{34}]NPY	4.4	3.5	115
h PYY	11	1.9	113
p PYY	4.1	1.3	67
b PP	1.8	>10000	165
h NPY-COOH	>10000	>10000	154
h NPY(1-24)	>10000	>10000	92
p NPY(25–36)	13	>10000	25
p NPY(18-36)	38	3200	23
[H^{32}L^{34}]NPY(32-36)	1000	1000	115
1229U91	2.3	1.9	103
Insect peptides			
Led-NPF1	45	>10000	>10000
Led-NPF2	40	>10000	>10000
Scg-NPF	0.6	>10000	>10000
Lom-MT1	40	>10000	>10000
Scg-MT2	78	>10000	>10000
Lom-PK2	15	>10000	>10000
Lom-MT3	1.8	>10000	>10000
Scg-MT1	88	>10000	>10000
Lom-MS	0.4	>10000	>10000

[a] [^{3}H]NPY was incubated with crude venom for 60 min at 30°C, with anti-NPY serum for 60 min at room temperature, and with CHO-Y_1 cells for 90 min at 37°C. All assays were performed in the presence of increasing concentrations of the tested peptides from 0.1 nM to 10 μM. Results are shown as the concentration (nM) of peptide and produces a 50% displacement (IC_{50}) of specific [^{3}H]NPY binding.

fragments of NPY produced only a partial displacement of [^{3}H]NPY binding to the antiserum. These peptides had IC_{50} values between 23 and 165 nM. As shown in Figure 3, incubation of the antiserum with a combination of NPY(1-24) and NPY(18-36) or NPY(25-36) at maximally effective concentrations (i.e. 10 μM) resulted in the complete displacement of [^{3}H]NPY binding. In the presence of NPY(1-24), Led-NPF1 and Led-NPF2 displaced [^{3}H]NPY binding with IC_{50} values greater than 10 μM (data not shown).

DISCUSSION

In mammals, NPY binds to at least six described subtypes of G-protein coupled receptors. These can be distinguished by their relative affinity for NPY, NPY analogs, non-peptide antagonists as well as other peptide members of the pancreatic polypeptide family (27, 28). It was found that a component of the *C. anemone* venom (referred to as ANPY toxin) binds directly and with high affinity (Kd in the nanomolar range), to the radioligand [^{3}H]NPY, but not to NPY-receptors of Y_1 and Y_2 subtypes. Specific binding of [^{3}H]NPY to ANPY toxin was completely inhibited by adding unlabeled NPY, PYY, PP as well as C-terminal NPY fragments (11). To explore the pharmacological characteristics of the ANPY toxin, we have now evaluated its capacity to bind a variety of mammalian and invertebrate neuropeptides. In addition, we have compared the binding properties of the ANPY toxin to these of Y_1 receptors and of a commercial anti-NPY antiserum.

The present study confirms that the ANPY toxin has high affinity to mammalian NPY and that the "epitope" which is recognized by ANPY is situated in the C-terminal portion of NPY. This is illustrated by the fact that the N-terminal NPY(1-24) fragment did not inhibit [^{3}H]NPY binding. Furthermore, the affinity of C-terminal NPY-fragments NPY(25-36), NPY(18-36) for ANPY was very similar to that of NPY itself. Since the amide function at the C-terminus of the peptide is very important for biological activity, it is not surprising that NPY-COOH did not bind with high affinity to ANPY and to Y_1 receptors. In the same line, the binding to the NPY-antiserum was also weaker for NPY-COOH than for the active, amidated form, but, as a result of the polyclonal origin of the antibodies, the difference in binding affinity was less pronounced (see

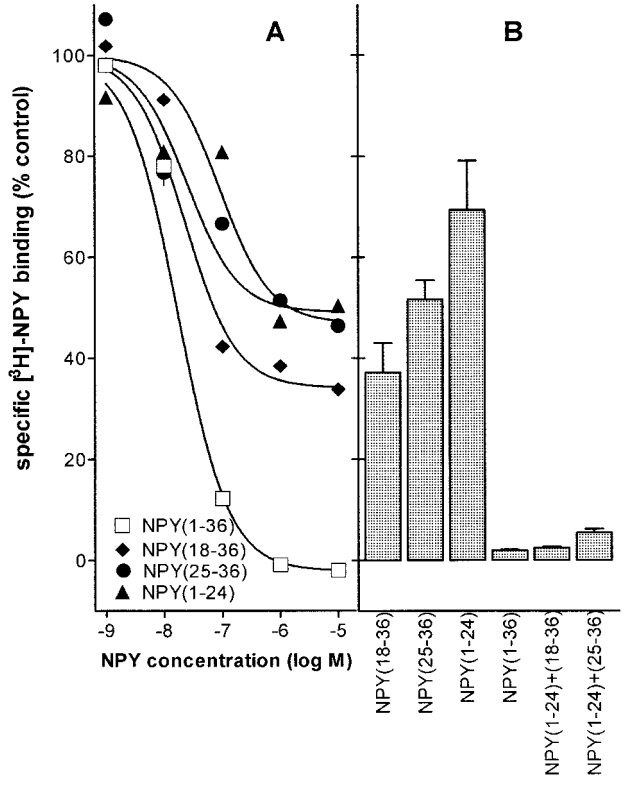

FIG. 3. Competition binding to anti-NPY serum. (A) Binding of [^{3}H]NPY in the presence of increasing concentrations of NPY and NPY fragments. (B) Binding of [^{3}H]NPY in the presence of 10 μM NPY(1-24), NPY(18-36), or NPY(25-36) either alone or in combination.

TABLE 2

Alignment of the C-terminus Peptide Sequences

Peptides	Amino acid sequences
Highly affinity binding compounds for ANPY	
Lom-MS	PDVDHVFL**R***F*a
Scg-NPF	YSQVARP**R***F*a
Lom-MT3	RQQPFVP**R**La
bovine PP	APLEPEYPGDDATPEQMAQYAAELRRYINMLTRP**RY**a
1229U91	2(IEPPYRL**RY**a)
porcine PYY	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQ**RY**a
[L^{31},P^{34}]NPY	YPSKPDNPGEDAPAEDLARYYSALRHYINLLTRP**RY**a
human PYY	YPIKPEAPGEDASPEELSRYYASLRHYLNLVTRQ**RY**a
human NPY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQ**RY**a
NPY(25-36)	RHYINLITRQ**RY**a
Lom-PK2	pQSVPTFTP**R**La
NPY(18-36)	ARYYSALRHYINLITRQ**RY**a
Lom-MT1	GAVPAAQFSP**R**La
Led-NPF2	APSLRL**R***F*a
Led-NPF1	ARGPQLRL**R***F*a
Scg-MT2	TSSLFPHP**R**La
Scg-MT1	GAAPAAQFSP**R**La
Weakly and inactive binding compounds for ANPY	
Scg-AST7	AGPAPSRLYSFGLa
Lom-AG-MT1	GFKNVALSTARG*F*a
[$H^{32}L^{34}$]NPY(32-36)	HRL**RY**a
Lom-MIP	AWQDLNAGWa
Pev-KIN1	ASFSPWGa
Corazonin	pQTFQYSHGWTNa
Lom-AG-MT2	AHRFAAEDFGALDTA.
Aea-TMOF	YDPAPPPPPP.
NPY-COOH	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQ**R**Y.
NPY(1-24)	YPSKPDNPGEDAPAEDMARYYSALa

Table 1). The modification of NPY at positions 31 and 34 ([$Leu^{31}Pro^{34}$]NPY) slightly improved its binding affinity for ANPY, suggesting that the modified sequence corresponds even better to the structural requirements needed for interaction with ANPY toxin. This was not the case for binding to the Y_1 receptors and the affinity of this analog for the antiserum was clearly lower than that of NPY. Furthermore, the cyclic peptide 1229U91, a C-terminal analog of NPY that was reported to be a potent Y_1 receptor antagonist (29, 30), displayed a similar, high affinity for ANPY and for Y_1 receptors. Even more striking was the discovery that two insect peptides, Lom-MS and Scg-NPF, had an affinity for the ANPY toxin which was more than 20 times higher than the affinity of NPY. Although these factors did not produce significant binding to Y_1 receptors or to the anti-NPY antibodies, they show sequence similarity with the C-terminal part of NPY. Among sixteen invertebrate peptides (Figure 2) that were tested, nine were binding to ANPY toxin with high affinity (IC_{50} in 0.1 nM–0.1 μM range). The high affinity of these peptides for ANPY toxin is probably due to their sequence similarity with the C-terminal part of NPY (20) or with the F-amide-like peptide (FLP or FaRP) superfamily, which is encountered in most invertebrate Phyla (31–33). In *C. elegans*, the only metazoan organism for which the entire genome information is known, at least 18 distinct genes, encoding more than 50 different FLPs, are found (34).

The pharmacological properties of ANPY clearly differ from those of the Y_1 receptors and of a polyclonal anti-NPY antiserum. This indicates that the optimal amino acid sequence for binding to ANPY differs from the one needed for interaction with Y_1 receptors or anti-NPY antibodies. A sequence comparison of all peptides that displayed high affinity binding to ANPY revealed that the C-terminal amide group and the arginine (Arg or R) residue at the penultimate position are probably the most essential elements responsible for a tight interaction with ANPY toxin (see Table 2). The final tyrosine (Tyr or Y) residue of NPY can be replaced by phenylalanine (Phe or F) or by leucine (Leu or L) without significant loss of affinity. The third last amino acid residue is often proline (Pro or P), leucine or glutamine (Gln or Q). As indicated above, a change of the glutamine residue of NPY into a proline even improved the affinity to ANPY. To gain further insight into the structural requirements that are needed for a strong binding to ANPY toxin, more analogs, eventually in the form of a random library of synthetic pep-

tides, will be screened. In addition, the affinity of restricted conformation analogs could be analyzed and the conformation of the epitope or motif that is recognized by ANPY could be further defined.

Anti-NPY antibodies have been produced and were shown to recognize different epitopes of NPY and to cross-react with NPY-related peptides (35, 36). The polyclonal anti-NPY serum that was used in this study appears to bind with high affinity to NPY and NPY fragments. It also cross-reacted with natural and synthetic NPY-analogs such as PYY, PP and 1229U91. The IC_{50} values of the truncated NPY-fragments NPY(1-24), NPY(18-36), and NPY(25-36) are similar to the IC_{50} of the intact NPY. However, a complete displacement of [^{3}H]NPY binding could not be obtained with these truncated fragments (Figure 3). This can be explained by the fact that the antiserum is polyclonal and, thus, consists of a mixture of antibodies recognizing various epitopes in the NPY molecule. This is confirmed by a binding experiment in which the competitive inhibition curves of the truncated fragments NPY(1-24), NPY(18-36) or NPY(25-36) were compared with the curves resulting from the combined fragments (Figure 3B). As predicted, the NPY(1-24) could fully compensate for the loss of binding activity of the C-truncated fragments.

In conclusion the venom derived from *Conus anemone* contains a soluble factor, ANPY, which possesses high affinity binding to the C-terminal part of NPY and to a number of invertebrate peptides. This compound is not the ligand for a pharmacological target in the prey, but it is an acceptor that binds with very high affinity a broad spectrum of neuropeptides that share a similar C-terminal amino acid sequence. Although it is not clear what might be the physiological effect of the ANPY toxin with respect to predation, this is a unique observation. The fact that the venom compound, ANPY, can interact with vertebrate as well as invertebrate peptides could help this *Conus* species to feed on "a broad spectrum" of preys, including worms, other molluscs, arthropods and small fish. Further experiments are underway to purify this binding factor, to analyze its physiological role and to find similar factors in other *Conus* species.

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