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Reduced Affinity of Iodinated Forms of Tyr^0 C-type Natriuretic Peptide for Rat Natriuretic Peptide Receptor B

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SUMMARY

Tyr^{0}CNP is an analogue of C-type natriuretic peptide (CNP) with a tyrosine residue added to the NH_2 terminus to allow its iodination. In the present study, the suitability of iodinated Tyr^{0}CNP as a ligand was tested, and its potency was compared with that of other natural rat natriuretic peptides or structural analogues by radioligand binding experiments. Binding studies were performed on membranes of COS-1 cells transfected with expression plasmids for either rat natriuretic peptide receptor (NPR)-A, rat NPR-B, or bovine NPR-C. ^{125}I-ANP(99–126) was used as a ligand to assess the binding characteristics of NPR-A and -C, and ^{125}I-Tyr^{0}CNP was used to study NPR-B. Binding associated to membranes of nontransfected COS cells was always <3% of the total binding observed in membranes from cells transfected with receptor expression plasmids. Receptor densities in transfected cells ranged from 500 to 2500 fmol/mg of protein. High performance liquid chromatography and ion-spray mass spectrometry analyses revealed that the reagents used in the course of iodination (lactoperoxidase, chloramine T, or *N*-chloromorpholine) altered the structure of Tyr^{0}CNP, most likely by changing the thiol of the Met^{17} residue into a sulfoxide. To further evaluate the usefulness of forms of iodinated Tyr^{0}CNP as radioligands, we performed iodination of the peptide with cold iodine (Na-^{127}I-). After purification by high performance liquid chromatography, three different modified peptides (i.e., Tyr^{0}Met(O)^{17}CNP, ^{127}I-Tyr^{0}Met(O)^{17}CNP, and $^{127}I_2$-Tyr^{0}Met(O)^{17}CNP) were recovered, and they were compared with CNP-22, Tyr^{0}CNP, ANP(99–126), BNP-32, and des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANP(4–23) NH_2 (c-ANP) for their ability to bind to transfected receptors. The binding affinity of Tyr^{0}CNP for NPR-A and -B receptors is similar to that of CNP. However, oxidation of the Met^{17} residue into methionine sulfoxide reduces the affinity of the compound for NPR-B by >10-fold, whereas the addition of one or two iodines did not further reduce its affinity. Similar results were obtained on evaluation of the ability of the oxidized form of monoiodinated Tyr^{0}CNP on the cGMP responses in cells transfected with NPR-B. In conclusion, the suitability of iodinated forms of Tyr^{0}CNP as radioligands for binding studies on rat NPR-B is not optimal, and the results of studies using such compounds for the detection, identification, and quantification of this receptor should be interpreted with caution.

The family of rat NPs comprises three different peptides that share ~60% homology in amino acid composition (1). They all feature a disulfide bond that forms a 17-amino acid ring structure, which is required for maximal expression of their biological activity. These peptides have been named ANP, BNP, and CNP, respectively. ANP is expressed mainly in the cardiac atria but is also found in lungs, kidneys, and pituitary gland and in the ventricles of the heart in the course of various cardiac pathologies (1). BNP is also expressed in the atria (although at a much lower level than ANP in the atria), and its synthesis is considerably augmented in the ventricles during heart failure (2). CNP is usually present in tissues at much lower concentration than ANP or BNP and is found exclusively in few tissues, such as brain and vascular walls, at higher concentrations than ANP (1). All three peptides have similar biological properties, i.e., they affect (although at varying degrees) sodium and water excretion, inhibit aldosterone secretion and renin release, relax vascular walls, and consequently lower blood pressure (2).

The effects of the NPs are mediated by extracellular membrane receptors distributed in various organs and tissues (3–6). These receptors have been cloned and classified as NPR-A, -B, and -C (4–6). NPR-A and -B are transmembrane receptors of ~120 kDa that possess an intrinsic intracellular guanylyl cyclase domain. cGMP is believed to be the second messenger responsible for mediation of most of the biological actions of NPs. NPR-A and -B share an overall identity of

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ABBREVIATIONS: c-ANP, des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANP(4–23) NH_2; ANP, atrial natriuretic polypeptide; BNP, brain natriuretic peptide; NPR, natriuretic peptide receptor; NP, natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; CMF-PBS, calcium/magnesium-free phosphate-buffered saline; HPLC, high-performance liquid chromatography; CNP, C-type natriuretic peptide.

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61%, the highest homology (88%) region being in the guanylyl cyclase domain and the lowest (44%) being in the extracellular domain. The third receptor, called NPR-C, has a molecular weight of 60 kDa and features an intracellular tail of only 37 residues that is devoid of any guanylyl cyclase activity. Its precise role is poorly understood, and although it has been proposed to act as a clearance receptor (5), it has been shown to mediate specific biological actions that may be linked to adenylyl cyclase and phospholipase C and A_2 activities (6, 7). Its extracellular binding domain shares only 33% and 30% identity with those of NPR-A and -B, respectively.

The affinities of ANP, BNP, and CNP for these various receptors have been determined by examining their relative ability to elicit a cGMP response in established cell lines or in cells transfected with NPR-A or -B (8–10), by comparing their ability to compete with the binding of labeled NP with membranes of cells transfected with plasmids coding for all three receptors (11), or by comparing their binding affinity for chimeric recombinant proteins consisting of the extracellular binding domains of the receptors fused to the Fc portion of human IgG (12). In addition, some investigations have compared the properties of receptors from different mammalian species. For example, Suga *et al.* (8) compared the relative affinities of receptor from human, bovine, and rat tissues or cell lines, and Schoenfeld *et al.* (9) compared the relative potencies of NPs on cGMP response in 293 cells transfected with either mouse, rat, or human NPR-A. Only minor differences have been observed among receptors from mouse, rat, and bovine species. However, human ANP is more specific on its cognate NPR-A than on any other receptor (8, 9). These experiments have also revealed that no single NP is absolutely specific for any kind of receptor. There is general agreement among studies that the potencies in competitive binding for NPR-A are ANP ≥ BNP > CNP, that they are CNP > ANP ≥ BNP for NPR-B, and finally ANP > BNP > CNP for NPR-C (1). However, radioligand binding techniques often failed to reveal the presence of NPR-B in rat cells that otherwise show a robust cGMP response on exposure to CNP and/or contain readily detectable NPR-B mRNA (8, 13, 14).

One potential problem in the latter studies may arise from the type of ligand that was used for binding. CNP lacks a tyrosine residue and cannot be easily labeled with radioactive iodine. Therefore, the Tyr^{0}CNP analogue is commonly used to generate radiolabeled ligand for binding studies and detection of NPR-B in slices or homogenates of tissues (12–19). The affinity of Tyr^{0}CNP for human recombinant NPR-B has been reported to be only slightly lower than that of CNP (11, 12). However, this may not be true with receptors from other species. For example, Gilkes *et al.* (15) reported that Tyr^{0}CNP was less potent than CNP in eliciting a cGMP response in mouse AtT-20 cells and that the response was even lower with ^{127}I-Tyr^{0}CNP. If iodinated Tyr^{0}CNP had a lower affinity than CNP for rat NPR-B, it might explain why many investigators have failed to identify this receptor in many rat CNP-responsive cells or tissues. To examine this question in detail, we compared the abilities of Tyr^{0}CNP and its iodinated forms with those of other NPs in terms of their ability to bind to membrane preparations of mammalian cells transfected with expression plasmids for various NP receptors. We focused our attention primarily on both types of rat guanylate cyclase-coupled receptors, i.e., NPR-A and -B. For comparative purposes, we also included NPR-C in our study.

Materials and Methods

Peptides, plasmids, and reagents. All rat peptides [ANP(99–126), BNP-32, CNP-22, Tyr^{0}CNP-22, cANP] were purchased from Peninsula Laboratories (Belmont, CA) or from Bachem California (Torrance, CA). Lactoperoxidase was obtained from Sigma Chemical Co. (St. Louis, MO). The expression plasmid pGEMAPNR3/4 [containing the entire coding region for bovine NPR-C (20) under the transcriptional control of the *SV40* promoter] was generously provided by Dr. J. Gordon Porter (Scios Inc., Mountain View, CA). The expression plasmids pCMV3/GC-A [containing the entire coding region for rat NPR-A inserted into the expression plasmid pCMV3 (21)] and pCMV5/GC-B [containing the entire coding region for rat NPR-B inserted into the expression plasmid pCMV5 (22)] were a generous gift from Dr. Stephanie Schultz (Howard Hughes Medical Institute, UTSMC, Dallas, TX). All plasmids were purified using Qiagen columns (Chatsworth, CA) before transfection.

Transfection of recombinant receptors in COS cells and preparation of membranes. COS-1 cells were seeded in 150-mm Falcon Petri dishes (Beckton-Dickinson, Oxnard, CA) at a concentration of 2×10^6 cells per dish, were grown overnight in growth medium [DMEM supplemented with 5% fetal calf serum] and then transfected with expression plasmids for either NPR-A, -B, or -C by the DEAE-dextran method, as described by Cullen (23). Briefly, the cells were rinsed with prewarmed CMF-PBS and then incubated with 2.5 ml of CMF-PBS supplemented with 25 μg of each plasmid and 0.7 ml of CMF-PBS solution containing 10 mg/ml DEAE-dextran (Sigma; molecular mass, ~500 kDa) for 30 min at 37°. Fifteen milliliters of an 80-μM solution of chloroquine in DMEM were then added to each plate, and cells were incubated for an additional 2.5 hr at 37°. The media were aspirated, and the cells were incubated for 2.5 min at room temperature in 10 ml of a solution of DMEM containing 10% dimethylsulfoxide. This solution was finally replaced with 20 ml of growth medium, and the cells were grown for another 48 hr before being harvested. For membrane preparations, the cells from each plate were washed twice with 5 ml of cold 0.05 M $NaHCO_3$, pH 8.3, and then scraped in the same buffer. The cells were homogenized with 10 strokes of a Teflon Potter-Elvehjem tissue grinder at 750 rpm, and the membranes were collected by centrifugation at 30,000 × *g* for 20 min. The pellet was dispersed in 0.05 M Tris·HCl, pH 7.4, and the membranes were aliquoted in small volumes and kept at −40° until use. Protein concentration in the homogenates was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as a standard.

Iodination of peptides. Radiolabeled peptides were prepared by the lactoperoxidase method in the presence of H_2O_2 and Na-^{125}I (24, 25). Purification of the monoiodinated peptides was made by reverse-phase HPLC on a C_{18} μ-Bondapak column (Millipore, Bedford, MA) with a 20–50% gradient of acetonitrile and 0.1% trifluoroacetic acid. This technique makes it possible to separate the noniodinated peptide from the monoiodinated and diiodinated forms. The specific activities of ^{125}I-ANP(99–126) and ^{125}I-Tyr^{0}CNP were assessed by the self-displacement method (using either anti-ANP antibodies or membrane preparations of cells transfected with NPR-B receptors) (26) and were found to average between 1500 and 2500 cpm/fmol.

Three different methods were used to perform iodination of Tyr^{0}CNP with cold iodine, i.e., the lactoperoxidase (24, 25), the chloramine T (27), and the *N*-chloromorpholine (28) methods. Because all three methods were found to cause oxidation of Tyr^{0}CNP (see Results), the chloramine T method was preferred when larger amounts of cold monoiodinated and diiodinated peptide were needed. Briefly, 84 nmol (200 μg) of Tyr^{0}CNP were added to 160 nmol of Na-^{127}I. The iodination process was started by rapid addition and vortexing of 400 nmol of chloramine T. After 90 sec, the incubation volume (1.12 ml) was rapidly injected on a C_{18} μ-Bondapak column, and the acetonitrile/0.1% trifluoroacetic acid gradient was run immediately. The 210-nm absorbency peaks were collected, and the peptides were analyzed by ion-spray mass spectrometry by Dr. Y.

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Konishi (Biotechnology Research Institute, Montreal, Canada). The amount of each peptide was estimated according to the integration of the absorbency peaks with Varian software of the Liquid Chromatograph Star Workstation (Varian Analytical Instruments, Sugar Land, TX) and calibration with known amounts of Tyr^{0}CNP. The total amounts of Tyr^{0}Met(O)^{17}CNP, ^{127}I-Tyr^{0}Met(O)^{17}CNP, and $^{127}I_2$-Tyr^{0}Met(O)^{17}CNP that were obtained in this fashion were 20, 38, and 28 nmol, respectively.

Binding assays. In competition experiments, 5–20 μg of membranes was incubated in a total volume of 0.25 ml of buffer containing 0.05 M Tris·HCl, pH 7.4, 0.5 mM $MgCl_2$, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% bacitracin, and 50 units/ml aprotinin in the presence of 80 pM of either ^{125}I-ANP(99–126) or ^{125}I-Tyr^{0}CNP and increasing concentrations (10^{-13} to 10^{-6} M) of the different unlabeled peptides. After 90 min of incubation at room temperature, the membranes were filtered on No. 34 glass-fiber filters (Schleicher & Schuell, Keene, NH) on a 30-well cell harvester (Brandel, Gaithersburg, MD) and washed twice with 4 ml of 0.05 M Tris·HCl, pH 7.4. The radioactivity on the filters was then measured in a γ-counter with 75% efficiency. For saturation experiments, the same protocol was used except membranes were incubated in the presence of increasing concentrations (0–1.6 nM) of the respective radioligands and in the absence of cold peptides. Nonspecific binding was determined in the presence of 10^{-6} M of the appropriate peptide.

Analysis of the data. The raw counts per minute of the saturation and competition experiments were analyzed with the computer software EBDA-Ligand version 2.0 (Biosoft, Ferguson, MO) as developed by McPherson (29). A one-site model was used when the cells were transfected with one receptor. The data were iteratively fitted as a nonlinear curve by minimizing the weighted sum of the square deviations, and the different models were then tested with a partial *F* test. The estimated final parameters (K_d and K_i) of three different experiments were averaged to calculate the mean ± standard deviation values. Comparison of the dissociation constants were made by one-way analysis of variance and the Student-Newman-Keuls test.

Generation and detection of cGMP in transfected cells. COS-1 cells were seeded into 150-mm dishes and transfected with NPR-B expression plasmids, as described above. Twenty-four hours later, they were passaged into 24-well plates and grown for an additional 24 hours. The cells were then rinsed with PBS supplemented with 0.5 mM isobutylmethylxanthine and then incubated for 10 min at 37° in DMEM, 10 mM HEPES, pH 7.4, containing 0.5 mM isobutylmethylxanthine and increasing concentrations of various NPs. The medium was then aspirated, and the cells were processed in 0.5 ml of cold 0.1 N HCl, as described previously (13). After 48 hr of incubation at 4°, precipitated proteins were separated by centrifugation. Aliquots of supernatant were acetylated for measuring the concentration of cGMP by radioimmunoassay as described previously (30, 31) using a specific rabbit antiserum developed in our laboratory against 2′0-succinyl cGMP. The amounts of cGMP generated in each well were then calculated according to the initial volume of extraction and corrected for the amount of DNA present in parallel wells of cells (measured by ethidium bromide fluorescence, as described previously [13]). These dose-response experiments were repeated three times with different batches of transfected cells, using triplicate wells for each concentration of peptide. To correct for differences in the efficiencies of transfection between experiments, the results were converted into percentage of maximal response by CNP (10^{-6} M), and the data from all three experiments were averaged.

Results

After iodinating Tyr^{0}CNP and purifying it by HPLC, the migration of the iodinated forms on reverse-phase HPLC was quite different from what could be expected. The peptide usually becomes more hydrophobic after the addition of one atom of iodine (and even more so when it is diiodinated) and therefore would elute much later. In the case of Tyr^{0}CNP, the iodinated peptides appeared to elute with a similar elution time as the intact peptide (Fig. 1). Moreover, a similar HPLC pattern was observed when the reaction was performed in the presence of only the oxidizing agent (i.e., H_2O_2) and in the absence of iodine (results not shown). This suggested that an amino acid residue might be oxidized in the course of iodination. One possible candidate was the methionine residue in position 17 of Tyr^{0}CNP. This amino acid is known as being very sensitive to oxidation, with the thiol group being transformed to a sulfoxide (32–34). Attempts to minimize the problem by using alternative iodination protocols were not successful because similar HPLC patterns were obtained when performing iodination with either chloramine T or even *N*-chloromorpholine (which has been suggested to be less deleterious to peptides as a function of its lower oxidoreduction potential [28]).

To analyze the iodinated forms of Tyr^{0}CNP in further detail, larger quantities of Tyr^{0}CNP were iodinated by the chloramine T method with cold iodine (Na-^{127}I). The resulting products were purified by HPLC. Three main peaks (A, B, and C) could be identified (Fig. 1). The peptides contained in each of these peaks were collected and further analyzed by ion-spray mass spectrometry (Table 1). The comparisons between the predicted theoretical molecular weight of various forms and the observed mass-to-charge ratio for peptides originating from the various peaks are presented in Table 1. This analysis revealed that methionine was oxidized in the peptides from all three peaks and that peaks B and C corresponded to the monoiodinated and diiodinated forms of the peptide, respectively. These peptides were used in competition studies to assess the binding properties of iodinated forms of Tyr^{0}CNP.

The NP receptors were transfected in COS-1 cells, and the membranes were prepared by homogenization and centrifugation. First, it was verified that either radiolabeled ANP or Tyr^{0}CNP did not show any significant binding to the membranes of wild-type COS cells compared with membranes of transfected cells. In all cases, binding associated to membranes of nontransfected COS cells was <3% of the total

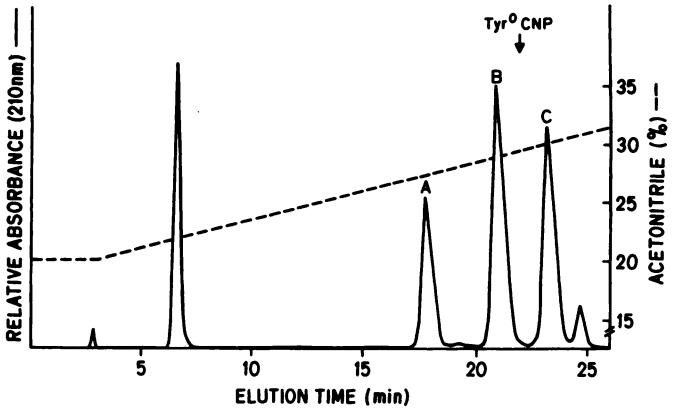

Fig. 1. HPLC elution pattern of Tyr^{0}CNP after cold iodination by the chloramine T method. Peaks A, B, and C were collected, and the material was analyzed by ion-spray mass spectrometry. *Arrow*, position of elution of Tyr^{0}CNP. The peak eluting at 6–7 min corresponds to chloramine T or its derivative.

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TABLE 1

Ion-spray mass spectrometry of the iodination products of Tyr^{0}CNP

Peak number	Peptide	Calculated molecular weight	Observed mass-to-charge ratio
Internal control	Tyr^{0}CNP	2359.1	2359.8
A	Tyr^{0}Met(O)^{17}CNP	2375.1	2375.8
B	^{127}I-Tyr^{0}Met(O)^{17}CNP	2502.0	2501.6
C	$^{127}I_2$-Tyr^{0}Met(O)^{17}CNP	2628.9	2627.8

binding of membranes from transfected cells and thus was considered to be negligible.

Affinities of the ^{125}I-ANP(99–126) and ^{125}I-Tyr^{0}CNP were determined by saturation analysis on membrane preparations of cells transfected for each type of NPR (results not shown). As predicted, ^{125}I-ANP bound best to NPR-A and -C, whereas ^{125}I-Tyr^{0}CNP bound mostly to NPR-B. Binding was found to be mathematically consistent with a one-site model. The affinity constants (as determined by Scatchard analysis; three experiments) of radiolabeled ANP were 91.4 ± 14.1 and 56.7 ± 16.8 pM for NPR-A and -C, respectively, and that of radiolabeled Tyr^{0}CNP was 220 ± 27 pM for NPR-B. The densities of the transfected receptors varied somewhat from one transfection to the other, but they were all in the range of 500-2500 fmol/mg of protein.

The specificity of each receptor was further assessed by competition analysis using the appropriate tracer and the following ligands, which were all of the rat species: ANP(99–126), BNP-32, CNP-22, Tyr^{0}CNP, Tyr^{0}Met(O)^{17}CNP, ^{127}I-Tyr^{0}Met(O)^{17}CNP, and $^{127}I_2$-Tyr^{0}Met(O)^{17}CNP. A synthetic ligand that is specific for NPR-C, i.e., c-ANP, was added to the list. Competition curves are shown in Figs. 2, 3, and 5, and calculated affinities are shown in Table 2. All the competition curves presented here were treated as a one-site model because only one receptor was transfected at a time and because there were only negligible amounts of wild-type receptors in nontransfected cells.

On transfected NPR-A (Fig. 2 and Table 2), the highest affinity was observed with ANP(99–126), followed by BNP, the group of CNP-related peptides, and, last, c-ANP. CNP was 2500 times less potent than ANP, and c-ANP was at least 25,000 times less efficient in displacing iodinated ANP. The addition of a tyrosine to the NH_2-terminal position of CNP had no effect on its binding properties, and further additions of one or two iodines on this tyrosine residue also did not affect it. Oxidation of the thiol group of Met^{17} into a sulfoxide decreases significantly its affinity, by 10-fold. However, it is surprising to observe that the same oxidation in the monoiodinated and diiodinated forms did not cause such an effect.

CNP was the most potent in displacing radiolabeled Tyr^{0}CNP from transfected NPR-B (Fig. 3 and Table 2). ANP and BNP bound to this receptor with affinities that were 250 and 900 times lower, respectively. The addition of a tyrosine residue in position 0 caused a significant 2-fold decrease in affinity, and oxidation of residue 17 reduced the affinity by an additional 10-fold. Cold iodination of the tyrosine in position 0 did not affect the binding any further. The synthetic ring-deleted peptide c-ANP was a very poor competitor of labeled Tyr^{0}CNP.

In parallel experiments, the potencies of Tyr^{0}CNP and ^{127}I-Tyr^0 Met(O)^{17}CNP were evaluated in terms of their ability to activate guanylyl cyclase of NPR-B and compared with the potency of other NPs (Fig. 4A). As expected, CNP was the most potent peptide, and minimal activation was obtained with either ANP or BNP. The addition of tyrosine in position 0 of CNP affected the activity of the peptide only minimally. Further addition of both one iodine on Tyr^0 and one oxygen on Met^{17} reduced the potency of the peptide by a factor of 10. An excellent correlation was observed between the affinities of the peptides for NPR-B and the EC_{50} values of their potency to elicit a cGMP response (Fig. 4B).

ANP was the best competitor of binding of ^{125}I-ANP on bovine NPR-C, was followed by c-ANP (Fig. 5 and Table 2). All other peptides were less active and almost equipotent. Compared with CNP, the addition of a tyrosine, the oxidation of the Met^{17}, and iodination of the molecule did not significantly alter the binding characteristic of the peptide.

Discussion

One of the first requirements for analysis of receptor binding is the availability of a ligand with high specificity and affinity. This ligand is then labeled with ^{3}H, ^{14}C, ^{125}I, or any fluorescent tag that does not interfere with its binding properties. Tritiated or ^{14}C compounds are in general best suited because the radiolabel is incorporated into the backbone of the peptide without causing modification of its structure. However, such labeled compounds also have drawbacks because their specific activity is often too low to be able to detect

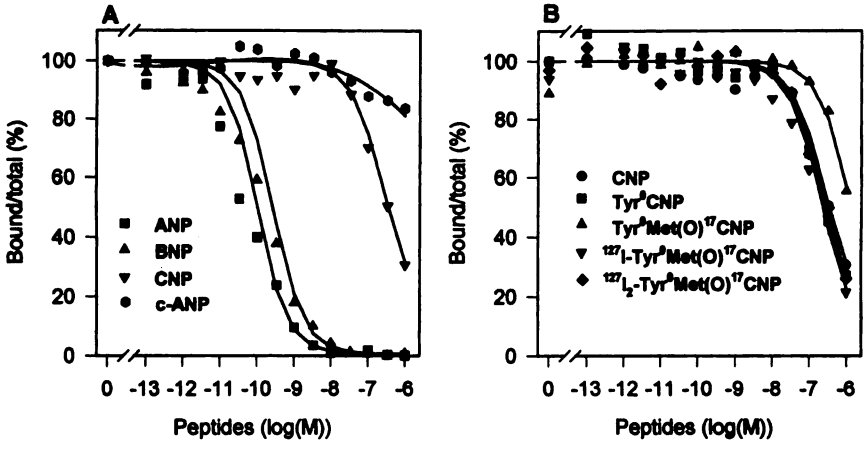

Fig. 2. Representative competition curves of NPs on membrane preparations of COS-1 cells transfected with rat NPR-A with ^{125}I-ANP(99–126) as radioligand. A, Competition curves of ANP(99–126), BNP, CNP, and c-ANP. B, Competition curves with CNP, Tyr^{0}CNP, Tyr^{0}Met(O)^{17}CNP, ^{127}I-Tyr^{0}Met(O)^{17}CNP, and $^{127}I_2$-Tyr^{0}Met(O)^{17}CNP. In all cases, maximal binding was expressed as 100%, and nonspecific binding was substracted.

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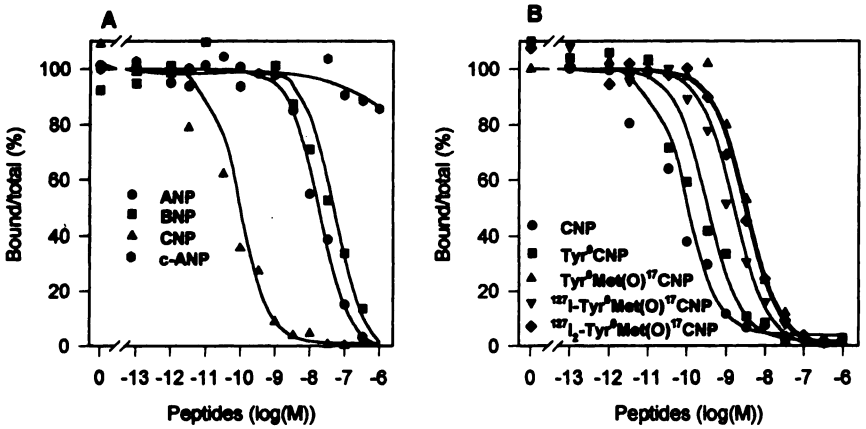

Fig. 3. Representative competition curves of NPs on membrane preparations of COS-1 cells transfected with rat NPR-B with ^{125}I-Tyr^{0}CNP as radioligand. A, Competition curves of ANP(99–126), BNP, CNP, and c-ANP. B, Competition curves with CNP, Tyr^{0}CNP, Tyr^{0}Met(O)^{17}CNP, ^{127}I-Tyr^{0}Met(O)^{17}CNP, and ^{127}I$_2$-Tyr^{0}Met(O)^{17}CNP. In all cases, maximal binding was expressed as 100%, and nonspecific binding was substracted

TABLE 2

Affinities (pK_i) of the rat natriuretic peptides on guanylate cyclase-coupled rat natriuretic peptide receptors and bovine clearance receptors

	NPR-A		NPR-B		NPR-C	
	pK_i	R.A.	pK_i	R.A.	pK_i	R.A.
ANP (99–126)	10.37 ± 0.14	1	8.00 ± 0.11[c]	0.0037	10.71 ± 0.22	1
BNP-32	9.82 ± 0.19[a]	0.28	7.46 ± 0.20[c]	0.0011	9.44 ± 0.13[a, •]	0.054
CNP-22	6.98 ± 0.05[a]	0.00041	10.43 ± 0.21	1	9.76 ± 0.21[a]	0.11
Tyr^{0}CNP	7.19 ± 0.09[a]	0.00066	10.12 ± 0.19[c]	0.49	N.D.	N.D.
Tyr^{0}Met(O)^{17}CNP	6.23 ± 0.11[a, b]	0.000072	8.96 ± 0.22[b, c]	0.034	N.D.	N.D.
^{127}I-Tyr^{0}Met(O)^{17}CNP	7.07 ± 0.06[a]	0.0005	9.22 ± 0.05[b, c, d]	0.062	9.33 ± 0.03[a, •]	0.042
^{127}I$_2$-Tyr^{0}Met(O)^{17}CNP	7.00 ± 0.17[a]	0.00042	8.71 ± 0.15[b, c]	0.019	N.D.	N.D.
C-ANP	>6[a]	<0.000043	>6[c]	<0.000037	9.93 ± 0.19[a]	0.162

R.A. = Relative affinity vs ANP on NPR-A or C or vs CNP on NPR-B.
N.D. = Not determined.
[a] $p < 0.05$ vs ANP.
[b] $p < 0.05$ vs Tyr^{0}CNP.
[c] $p < 0.05$ vs CNP.
[d] $p < 0.05$ vs ^{127}I$_2$-Tyr^{0}Met(O)^{17}CNP.
[•] $p < 0.05$ vs C-ANP.

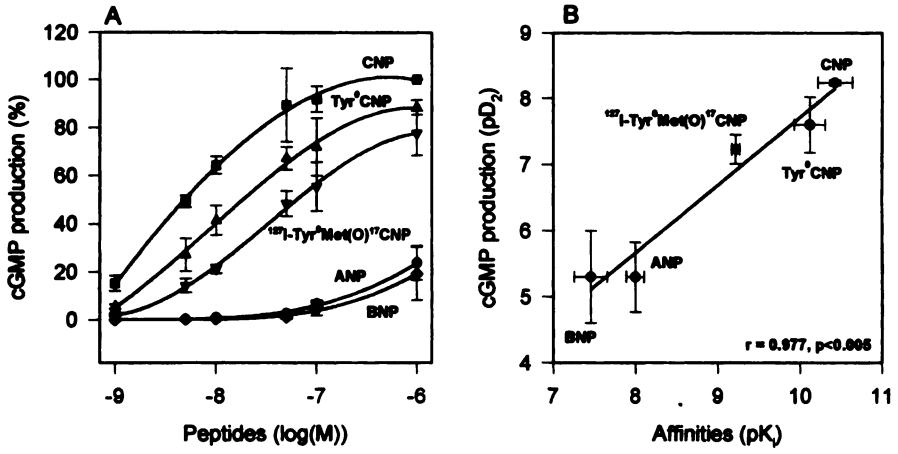

Fig. 4. A, Stimulation of cGMP production by increasing concentrations of NPs in cultured COS-1 cells transfected with rat NPR-B. *Curves*, mean ± standard error of three experiments, and the results were expressed by comparison to maximal stimulation at 10^{-6} M CNP, which was fixed at 100%. B, Correlation between affinities (pK_i values of Table 2) of NPs on transfected NPR-B and calculated concentrations of NP, which gives 50% of stimulation (pD_2) in Fig. 4A. The pD_2 values were calculated by logit-log transformation of the data of individual experiments.

and characterize low concentrations of receptors. Therefore, a commonly used alternative is to label peptides with radioisotopes of iodine. Although this technique yields labeled molecules of very high specific activity, it also has some disadvantages: the only residues susceptible to be iodinated are tyrosine and, to a lower extent, histidine. In addition, iodine is a bulky atom that can disturb the tridimensional structure of the compound or cause a steric hindrance that interferes with the binding of the ligand to its receptor site. The problem is sometimes proportional to the number of iodine molecules that can be added to the peptide. For example, the monoiodinated form of ANP(99–126) is a good ligand for its receptor, whereas the affinity of the peptide decreases by a factor of 3–4 when two atoms of iodine are incorporated (24). One final drawback is that the chemical reaction of iodination requires the use of reagents that have a high oxidoreduction potential and carry the risk of altering the molecule in other places.

CNP, unlike ANP(99–126), does not harbor any tyrosine residue. A common alternative is therefore to use a CNP peptide with a tyrosine residue added to the NH_2 terminal. In the present work, we report that addition of tyrosine mole-

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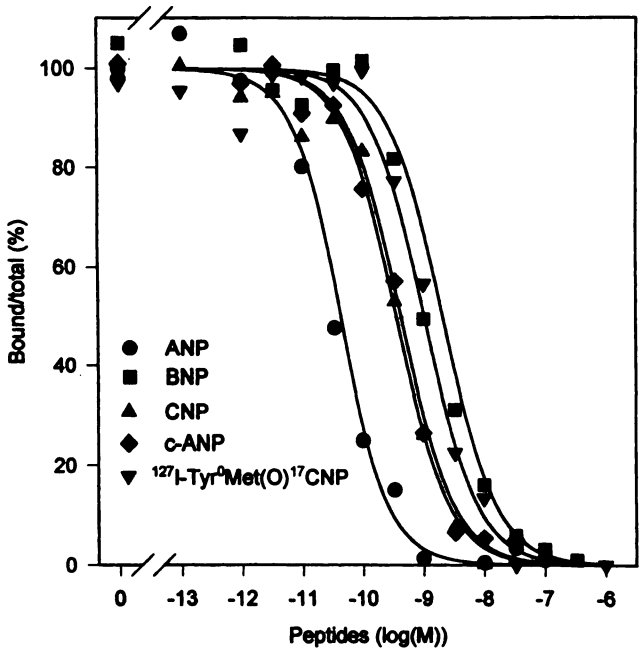

Fig. 5. Representative competition curves of NPs on membrane preparations of COS-1 cells transfected with bovine NPR-C with ^{125}I-ANP(99–126) as radioligand. Competition curves of ANP(99–126), BNP, CNP, c-ANP, and ^{127}I-Tyr^{0}Met(O)^{17}CNP. In all cases, maximal binding was expressed as 100%, and nonspecific binding was substracted.

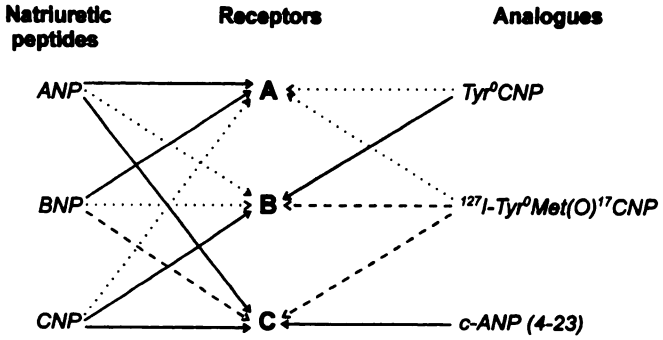

Fig. 6. Schematic representation of the affinities of the different NPs used in the study toward the NPR-A, -B, and -C. Affinities have been classified as very good (*solid line*, <10-fold decrease in affinity), fair (*dashed line*, 10–100-fold decrease in affinity), or poor (*dotted line*, >100-fold decrease in affinity) relative to the peptide was the highest affinity.

cules *per se* does not much affect the binding properties of the peptide compared with CNP but that (regardless of the method that was used) iodination always generated compounds with patterns of elution on HPLC that were unusual. Molecular mass of the peptide recovered from each HPLC peak revealed by ion-spray mass spectrometry that the mass of each peptide was augmented by a factor that corresponded in part to one or two iodine(s) but also probably to an atom of oxygen. The only plausible explanation for this phenomenon is that the thiol group present in the methionine residue in position 17 is oxidized. This group is well known to be very sensitive to oxidation, particularly in the presence of the oxidative agents used for iodination (32–34). One possible way to overcome the oxidation of methionine would be to reduce it with mercaptoethanol after iodination of the peptide, as has been proposed for substance P (35). However, this strategy is not applicable to CNP, as it would open the disulfide bridge that is required for its biological activity (36). We therefore proceeded to verify whether the iodinated oxidative forms of Tyr^{0}CNP could constitute acceptable ligands.

The relative affinities of the natural NPs, of Tyr^{0}CNP, of the monoiodinated oxidized forms of Tyr^{0}CNP, and of the ring-deleted analogue c-ANP for each type of receptor are schematically compared in Fig. 6 and can be summarized as follows: NPR-A, ANP ≥ BNP >> CNP; NPR-B, CNP >> ANP ≥ BNP; and NPR-C, ANP ≥ CNP ≥ BNP.

The rank-order of potencies of the natural NPs in competitive binding for the various receptors confirm and extend data that had been reported previously with different models, i.e., fusion proteins consisting of the human extracellular domain of the NPRs linked to an Fc fragment of IgG (12), membranes from cells transfected with the extracellular domains of each receptor (11), or membranes from either tissues or cell culture from different species (8). In particular, CNP is most specific for NPR-B, and the affinities of BNP are very comparable to those of ANP on each kind of receptor. c-ANP appears to be very specific for NPR-C, as it will interact with guanylyl cyclase-linked receptors only at concentration of $>10^{-8}$ M. Finally, Tyr^{0}CNP has a specificity that is similar to that of CNP, i.e., it is a very good ligand for NPR-B but a poor ligand for NPR-A. However, iodination and oxidation of the molecule reduce considerably its binding capacity, so that it can only be considered a "fair" ligand. Of note, a similar observation has been made previously with human ANP(99–126) (37, 9). This peptide possesses a methionine in position 110 (instead of the isoleucine in the rat sequence), and oxidation of the thiol group leads to a 20–30-fold decrease in its biological properties.

The effect of iodination plus oxidation of Tyr^{0}CNP was restricted to its affinity for NPR-B and had very few effects on the affinity of the peptide for other NPRs. Thus, all iodinated forms of Tyr^{0}CNP had potencies similar to that of CNP in competitive binding for NPR-A, although none of the forms of CNP-derived peptides bound to this receptor with high affinity. Similarly, the affinity of iodinated Tyr^{0}CNP for NPR-C was not much different from that of CNP.

Of note, we did use cells transfected with the bovine form of NPR-C. More recently, the sequence of rat NPR-C has been reported (38). One might expect the binding properties of bovine and rat NPR-C to be similar because both receptors display 94% homology in amino acid sequence (38). However, this should not be taken for granted because it has been shown that the pharmacology of human NPR-C (versus that of rat or bovine NPR-C) may be affected by a single amino acid substitution (Ala^{188} to Ile^{188}) (38). Nevertheless, both rat and bovine receptors share the same alanine residue in position 188. Furthermore, Suga *et al.* (8) have shown that bovine and rat NPR-C (in contrast to human NPR-C) have the same affinity for CNP.

Recent work by Schoenfeld *et al.* (9) has shown that human natriuretic receptors have a higher affinity for their natural ligands than for NPs from any other species. For example, human NPR-A was fully activated by either human ANP(99–126) or human BNP but only partially activated by the rat counterparts. The same problem was observed when rat and human peptides were assayed on rat NPR-A. This example and the present study stress the need of evaluating the

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receptors of one species with the corresponding peptides and may explain some divergent results in the literature.

Of note, no single ligand is able to detect simultaneously all three receptors. Numerous studies on the detection of the NPRs by autoradiography or by radioligand binding analysis were carried out with radioiodinated ANP using concentrations in the range of 10–100 pM (e.g., see Refs. 18 and 39–41). Considering the affinity of ANP to NPR-B (pK_i = 8.00), the radioligand will either fail to detect or bind to only <5% of the NPR-B present in the preparation at these concentrations. Therefore, investigations with labeled ANP probably underestimate the presence of NPR-B in tissue slices or homogenates. Our data indicate that the same problem may be encountered with radioiodinated Tyr^0CNP because its iodination causes a chemical modification that decreases its affinity for its cognate receptor. Consequently, investigations carried out with concentrations of iodinated Tyr^0CNP ranging from 50 to 100 pM may also have underestimated the binding densities of NPR-B (e.g., see Refs. 14 and 15).

In summary, our data provided a comprehensive and systematic investigation of the specificities of various forms of rat NPs for both forms of recombinant rat guanylate cyclase-coupled NP receptors. Previous studies had been conducted with various preparations of mostly human receptors, and it is becoming increasingly apparent that such data cannot be automatically extended to rat receptors. Because rat is a widely used experimental model, our data provide much needed information for the interpretation of binding studies in this species. Most important, we showed that iodination of Tyr^0CNP yields an oxidized peptide that has a reduced ability to bind to NPR-B and that the suitability of such compounds for binding studies is not optimal.

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