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Development of *p*-Benzoylbenzoylated [N,C,rANP(1-28)]pBNP32 (pBNP1) Derivatives and Affinity Photolabeling of the Bovine NPR-A Receptor

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Two N^{α}-benzophenone-substituted photoprobes, derived from the high affinity NPR-A chimeric agonist [N, C, rANP(1-28)]pBNP32 (pBNP1) were assembled by solid-phase peptide synthesis. [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (probe A), and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 (probe B) were synthesized and their affinity was tested on bovine zona glomerulosa membrane preparations. Both were found to exert ANP-type high affinities (K_d = 20 pM) with K_d of 10 pM and 30 pM for probe A, and probe B, respectively. Photolabeling of NPR-A with both analogs cross-linked specifically the 130 kDa monomeric NPR-A. The maximal irreversible ligand incorporations were estimated at 18% and 41% for probe A, and probe B, respectively. These results show that the N-terminus of the chimeric compound can be acylated with a large chemical function, such as the benzophenone moiety, without loosing its affinity for the NPR-A receptor. Furthermore, Leu^{2} or Leu^{18} can be substituted with tyrosine without disturbing the binding capacity of the ligand. Finally, it appears that the pBNP1 N-terminus is close to the receptor structure as irreversible incorporation is observed after photolabeling.

Signal transduction through ligand-induced stabilization of homodimeric cell surface receptor complexes is a well established regulation mecanism. In such cases as the human growth hormone or erythropoietin, crystallographic data have shown that the ligand was in fact interacting with both subunits of their receptor. In accordance with the dimeric nature of both the growth hormone receptor and the soluble guanylyl cyclase, NPR-A was shown by steric exclusion chromatography (1) and by chemical cross-linking experiments (2) to exist as a homodimer or a homotetramer. Comparison of the measured number of ANP binding sites assessed by saturation binding curves with immunoassayable dosage of receptor protein revealed a stoichiometry of one molecule of ANP per receptor dimer (3). Recently, the labeling probe [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (4), which permitted identification and localization of a portion of the NPR-A binding domain (5), was derived into the bifunctional probe [*p*-azidobenzoyl-Ser^{1}, Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (3). Photoaffinity labeling of bovine adrenal *zona glomerulosa* NPR-A with this bifunctional probe provided evidence that the amino- and carboxy-terminal ends of ANP are adjacent to distinct subunits of the receptor dimer, and that the amino-terminal end is in fact interacting with one of the subunits. Nevertheless, the poor efficiency of the photolabile agent used (*p*-azidobenzoyl-) for the N^{α} derivation of this bifunctional probe made difficult the full assessment of the results obtained.

Therefore, we pursued the evaluation of the purported interaction of the natriuretic peptide exocyclic N-terminal segment with the NPR-A receptor by developing two N^{α}-benzophenone derived photoaffinity labeling probes (Figure 1). Poor specificity and incorporation efficiency of the azido group (1%–2%) (6) were circumvented by replacing it with the highly site specific and efficient benzophenone (7). Furthermore, the high affinity 28-residue hybrid natriuretic analog [N,C,rANP(1-28)]pBNP32 (K_d = 8 pM) (8) obtained by combining the ring structure of pBNP32 (K_d = 36 pM)

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Abbreviations: ANP, atrial natriuretic peptide; Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; NPR-A, natriuretic peptide receptor type A; pBNP32, porcine brain natriuretic peptide; pBNP1, [N,C,rANP(1-28)]pBNP32; Probe-A: [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1; Probe B: [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1.

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Peptide	Structure
rANP(1-28)	SLRRSSCFGGRIDRIGAQSGLGCNSFRY
pBNP32	SPKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
pBNP1	SLRRSSCFGRRLDRIGSLSGLGCNSFRY
[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1	*p*-benzoylbenzoyl-S<u>Y</u>RRSSCFGRRLDRIGSLSGLGCNSFRY
[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1	*p*-benzoylbenzoyl-SLRRSSCFGRRLDRIGS<u>Y</u>SGLGCNSFRY

FIG. 1. Primary structures of rANP(1-28), pBNP32, the related chimeric analog pBNP1, and the two synthesized photoaffinity labeling probes. Substituted amino acid residues found in both photoprobes, in comparison with pBNP1, are underlined.

with the amino- and carboxy-terminal portions of rANP(1-28) (K_d = 20 pM) was used as the template in order to highly favor the affinity of the designed labeling probes. Finally, a tyrosine substitution was made in order to track proteolytic fragments (Figure 1). Substitution L2Y and L18Y were made according to structure-activity relationship studies. It is of general agreement that ANP, BNP, and pBNP1 all share a similar internally driven hydrophobic core formed by the side-chain clustering of Phe^{8}, Leu^{12}, Ile^{15}, and Leu^{21} (9, 10). Single substitution experiments of ANP(3-28) produced low potency analogs when Phe^{8}, Ile^{15}, or Leu^{21} were replaced by alanine residues (11). Similar experiments done on hANP have determined the prime importance of Phe^{8}, Asp^{13}, Arg^{14}, Ile^{15}, Leu^{21}, and Arg^{27} (12). The same residues were found important when an alanine scan was performed on rANP(3-28) (11), and as such were not tempered with. Nor were the residues Arg^{3}, and Arg^{4} for reasons stated above. Choice of Leu^{2} and Leu^{18} for tyrosine substitution sites was also based on surface accessibility shown by NMR molecular modeling of pBNP1 (9) and on the NMR modeling of the ANP hexamutant ([R3D, G9T, R11S, M12L, R14S, G16R]hANP(1-28)) (10). The hydrophobic nature of both residues was also considered favorable.

Thus we report the design of two high affinity labeling probes [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 and show that these derivatives display excellent incorporation efficiency with the NPR-A receptor, as compared to cross-linking or affinity cross-linking equivalents.

METHODS

Peptide synthesis. All analogs were synthesized using the solid-phase peptide synthesis strategy (13) combined with the Boc chemistry methodology. The peptides were assembled using a homemade manual multireactor system and BOP (Richelieu Biotechnologies) was used as a condensating reagent. The coupling reactions were monitored with the ninhydrin test (14). The final N$^{\alpha}$ derivatization was achieved using the BOP-driven coupling of *p*-benzoylbenzoic acid (Sigma Ltd). Amino acid side-chain deprotections, as well as peptide cleavages from the solid support were obtained with liquid hydrogen fluoride in presence of *m*-cresol (1 ml/g) at 0°C for 90 min. After precipitation and washings with anhydrous diethylether, the crude peptides were extracted with pure trifluoroacetic acid (Halocarbon) followed by evaporation.

Peptide purification. Analog purification was performed by preparative HPLC using a DeltaPak C_{18} (15 μm, 30 nm) reverse-phase column (30 cm x 4.7 cm) connected to a Waters Prep LC 500A system equipped with a Waters 1000 Prep Pak Module and a 441 absorbance detector. A standard binary elution system with (A) 0.06% TFA/H_2O, and (B) acetonitrile (35%) in 0.06% TFA/H_2O was used. Detection was performed at 230 nm. Fractions (5 ml) were analyzed by analytical HPLC on a 600 Multisolvent delivery System equipped with a Waters 484 Tunable Absorbance Detector Spectrophotometer. The analyses were carried out using a Vydac C_{18} (5 μm) reverse-phase column (30 cm x 0.39 cm) in combination with a binary elution system of (A) 0.06% TFA/H_2O, and (B) acetonitrile. Detection was performed at 230 nm.

Preparation of membranes. Bovine adrenal *zona glomerulosa* membranes were prepared as previously described (15) from kidney obtained from a local slaughterhouse. Briefly, 0.5 mm layers of cortex, corresponding to the *zona glomerulosa* were dissected and homogenized in buffer containing 20 mM sodium bicarbonate, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin A, and the membrane pellet was washed twice with the same buffer. Membranes were frozen in liquid nitrogen and stored at −80°C until used. Protein concentration in membrane preparations was determined using the BCA protein assay kit from Pierce.

Binding assays. Bovine adrenal *zona glomerulosa* membranes corresponding to 20 μg of protein were incubated with 10-20 pM of ^{125}I-rANP(1-28) and varying concentrations of the unlabeled competing analog. The experiment was carried out at ambient temperature for 90 min in 1 ml of buffer (50 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 0.5% bovine serum albumin, and 5 mM magnesium chloride). Bound

^{125}I-rANP(1-28) was separated from free ligand using a cell harvester by filtration through Whatman GF/C filters pretreated with 1% polyethyleneimine. The filters were counted for radioactivity in a LKB 122 CliniGamma counter with 70% efficiency. In the case of saturation binding assays, membrane preparations corresponding to 20 μg of protein were incubated with varying concentrations of the labeled analog with or without saturating concentration (10^{-7} M) of unlabeled rANP(1-28). Experimental conditions were the same as those described above.

Iodination of analogs. Iodinations were performed with lactoperoxydase. Briefly, the peptide (6 nmoles) was dissolved in 20 μl of a 100 mM sodium acetate buffer, pH 5.6, and incubated in the presence of 1 mCi of carrier-free Na^{125}I (Amersham Life Science Inc.) and 100 ng of lactoperoxydase. The enzymatic reaction was started with the addition of 5 μl of an aqueous solution of H_2O_2 (1:15,000), and was maintained with two more additions at 5 and 10 min. After a total time of 15 min, the reaction was stopped with 1 ml of trifluoroacetic acid 0.1%. The solution was filtered on a 0.45 μm nylon 66 Conz Syringe-Tip filter, and the iodinated peptide purified on a Vydac C_{18} (10 μm) reverse-phase column (30 cm x 0.39 cm). Elution was achieved at a flow rate of 1 ml/min with a 80-min linear gradient at 15%–55% acetonitrile 90% in 0.1% trifluoroacetic acid. Fractions of 1 ml were collected and counted for radioactivity. The theoretical specific activities of the monoiodinated derivatives was set at 2000 Ci/mmol.

Analogs digestion with carboxypeptidase A. Approximately 400 fmoles of iodinated peptide were dissolved in 1 ml of a 200 nM ammonium bicarbonate buffer, pH 8.5 in presence of approximately 25 ng of carboxypeptidase A (Boehringer Mannheim). At the end of a 30 min incubation at 37°C, the reaction mixture was filtered through a Waters C_{18} Sep-Pak cartridge, washed with 3 x 1 ml fractions of trifluoroacetic acid 0.1%, and then eluted with 3 x 1 ml of 45% acetonitrile. Fractions were counted for radioactivity.

Photoaffinity labeling with iodinated analogs. Photoaffinity labeling was achieved as previously described (4). Radioligand binding was achieved as stated above. Tubes were purged with helium and placed on ice at a distance of approximately 8 cm from two mercury lamps (54 mW/cm^2 at 365 nm) for a 15 min irradiation. Tubes were then centrifuged at 40,000 x g for 30 min. The pellets were resuspended in buffer containing 50 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, and centrifuged a second time. The pellets were dissolved in a sample buffer containing 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercaptoethanol, and 0.05% bromophenol blue, and their protein content was analyzed by SDS-PAGE. Autoradiographic exposure was done at −80°C with KodaK X-Omat XK-1 film with two intensifying screens.

Data analysis. Competitive binding curves and receptor saturation curves were analyzed by weighted least-square regression using the computer program ALLFIT based on a generalized form of the law of mass action (16).

RESULTS

Peptide synthesis and purification. Probes A and B (Fig. 1) were synthesized using BOP as the coupling reagent (17) according to previously described synthetic methods (18). The incorporation of the photophore was achieved by carrying out a N-terminal derivatization with BOP as the coupling reagent and the commercially available *p*-benzoylbenzoic acid. This procedure avoided the use of the expensive synthetic amino acid *p*-benzoyl-L-phenylalanine. Similar strategies have been reported using dicyclohexylcarbodiimide as the coupling reagent (19, 20). Purification was achieved using preparative HPLC, and isolated fractions were monitored by analytical HPLC. Purity of the crude material ranged between 13%–19%. Highly purified (≥98%) preparations of linear analogs were obtained as confirmed by 5 μm reverse-phase analytical HPLC, prior to cyclization. Peptide cyclization yields ranged between 17%–20%. Purity of the cyclic analogs (≥98%) was assessed by analytical HPLC, capillary electrophoresis, and by electrospray mass spectrometry (m/z 3421.2 ± 0.5 and 3421.5 ± 0.6 for probe A, and probe B, respectively; expected: 3420.9 in both cases).

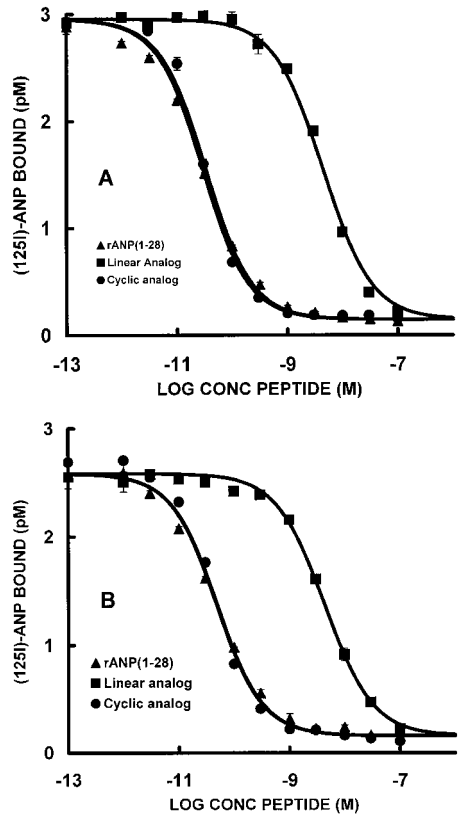

FIG. 2. Competition binding curves of control rANP(1-28) and the two analogs in both their linear and cyclic forms for the binding of ^{125}I-rANP(1-28) to NPR-A from bovine zona glomerulosa membrane preparations. Membrane protein (20 μg) was incubated 90 min at room temperature with approximately 10 pM of ^{125}I-rANP(1-28), and increasing concentrations of the competing unlabeled peptides in their respective cyclic and linear form. Radioligand binding measured after separation and washing on GF/C filters is reported for [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^2]pBNP1 (**A**), and [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, (**B**). Curves (n = 3) were analyzed by the computer program ALLFIT for nonlinear least-square regression based on a generalized form of the law of mass action (De Léan *et al.*, 1982).

Binding assays. Bovine adrenal *zona glomerulosa* membrane preparations were used to assess the potencies of both analogs. This tissue provides a simple, sensitive and reliable model which has been found to be rich in the NPR-A receptor subtype (21). Affinities were determined by competitive binding assays against ^{125}I-rANP(1-28). As shown in Fig. 2, both ana-

TABLE 1

NPR-A Affinities of rANF(99-126), [N^{α}-*p*-Benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-Benzoylbenzoyl, Tyr^{18}]pBNP1 in Bovine Adrenal Zona Glomerulosa Membrane Preparations

Peptide	pK_i		Slope factor	
	Linear peptide	Cyclic peptide	Linear peptide	Cyclic peptide
rANP(1-28)	N.D.	10.71 ± 0.10	N.D.	N.D.
pBNP1	N.D.	11.26 ± 0.07	N.D.	N.D.
[N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1	8.70 ± 0.30	10.98 ± 0.11	1.1	1.3
[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1	8.53 ± 0.06	10.53 ± 0.11	1.0	1.1

Note. Affinities are given as pK_i ($-\log K_d$); mean ± standard error of two to four receptor binding assays with ^{125}I-rANF(99-126).

logs were able to compete against ^{125}I-rANP(1-28), displaying competition binding curves with slope factor close to unity (Table 1), as expected with a model involving a single class of receptor sites. As shown by the pK_i values in Table 1, both analogs in their linear form exhibit very low potencies relative to rANP(1-28) (≤1%), stressing the importance of the disulfide-bridge (8). As for the cyclic analogs, both peptides displayed ANP-like affinities with pK_i values of 10.98 ± 0.11 and 10.53 ± 0.11 for probe A and probe B, respectively, as compared to 10.71 ± 0.10 for rANP(1-28) (Table 1).

Iodination of analogs. Purification of probe ^{125}I-A and probe ^{125}I-B yielded radioligands with specific activities around 2000 Ci/mmol. Both labeled analogs eluted at a 2% higher concentration of acetonitrile as compared to their unlabeled form, guaranteeing good segregation of both species. Double iodination was minimal despite the fact that both analogs contain two tyrosine residues. Purified iodinated fractions were checked in order to estimate the propensity of the Tyr residues to be preferentially targeted in the iodination process. Analysis was performed on iodinated analogs after a carboxypeptidase A treatment producing a cleavage of the C-terminal tyrosine residue. The labeled parent compound pBNP1 which possesses only one tyrosine residue located at the C-terminal served as a control. Results indicate that the iodination process is highly preferential toward the C-terminal tyrosine. This residue exhibited 80% and 90% of the radioactivity measured for probe ^{125}I-A, and probe ^{125}I-B, respectively.

Specific binding of analogs to NPR-A. Both analogs display significantly higher non-specific binding as compared to the parent compound pBNP1. While non-specific binding for pBNP1 corresponds to only 3% of the total bound ligand, the ratio of nonspecific binding divided by free ligand soars to 34% for probe ^{125}I-A, and to 22% for probe ^{125}I-B, as computed by analysis of the saturation curves (Figs. 3 and 4).

Photoaffinity labeling of NPR-A with iodinated analogs. Both photoaffinity labeling probes specifically cross-linked the M_r 130 kDa NPR-A monomeric form (Fig. 5). Maximum yield of incorporation for probe ^{125}I-A (≈1700 cpm/20 μg of protein) was set at 18% of the total specifically bound radioligand. Maximum yield of incorporation for probe ^{125}I-B (≈3300 cpm/20

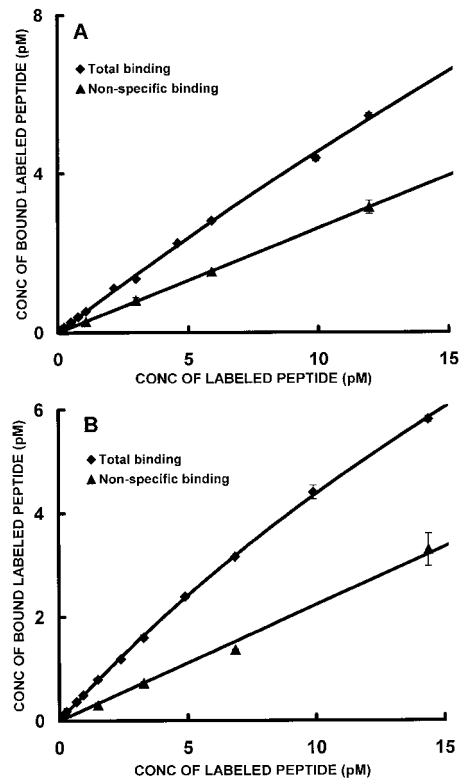

FIG. 3. Saturation binding curves of ^{125}I-[N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and ^{125}I-[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 obtained with the NPR-A receptor found in bovine zona glomerulosa membrane preparations. Membrane protein (20 μg) was incubated 90 min at room temperature with increasing concentrations of the labeled analog with or without saturating concentration (10^{-7} M) of unlabeled rANP(1-28). Radioligand binding measured after separation and washing on GF/C filters is reported for cyclic [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (**A**), and cyclic [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]-pBNP1, (**B**). Curves (n = 3) were analyzed by the computer program ALLFIT based on a generalized form of the law of mass action (De Léan *et al.*, 1982).

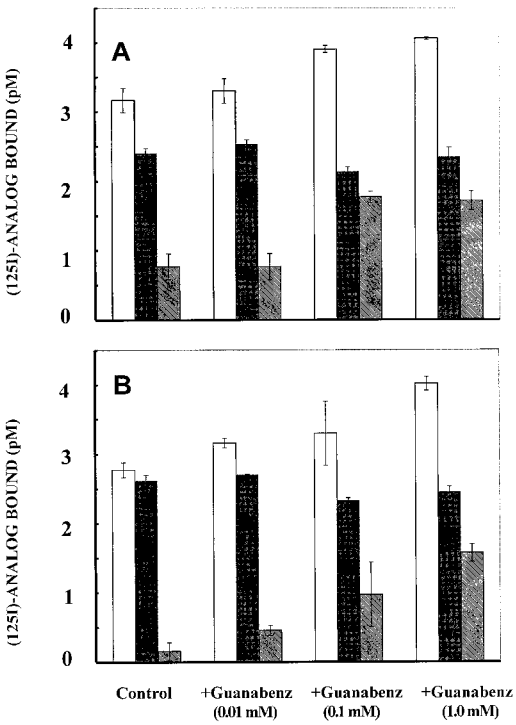

FIG. 4. Influence of the antihypertensive agent guanabenz in the augmentation of specific binding of ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]-pBNP1, and ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1. Bovine adrenal zona glomerulosa membrane protein (20 μg/ml) was incubated without (control) and with increasing concentrations of guanabenz (n = 2). Total radioligand binding (□), non-specific binding (■), and specific binding (▩) measured after separation and washing on GF/C filters are reported for cyclic [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (**A**), and [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, (**B**).

μg of protein) was set at 41% of the total specifically bound radioligand.

DISCUSSION

We have synthesized the photoaffinity probes ^{125}I-A, and ^{125}I-B using the BOP reagent for the coupling of the Boc-amino acids and the N-terminal photoreactive moiety *p*-benzoylbenzoic acid. These two synthetic peptides displayed high affinities toward the NPR-A receptor with estimated K_d of 10 pM and 30 pM for probe-A, and probe-B, respectively. As compared to the parent compound pBNP1 (K_d = 8 pM) only a little loss of affinity was measured following the chemical substitutions. Furthermore, these affinities are in the same order of magnitude than that of ANP (K_d = 20 pM) obtained in the same experimental conditions. The use of the high affinity chimeric analog pBNP1, as a template for the development of photoaffinity labeling probes, is therefore suitable. The incorporation of a benzophenone group yielded species with enhanced hydrophobic character. Both analogs eluted on a C_{18} reverse-phase column at a 5% higher concentration of acetonitrile than the parent compound pBNP1, and at a 7% higher concentration of acetonitrile when iodinated. In saturation experiments, it appears that this increased hydrophobicity translated in high non-specific binding corresponding to 34% and 22% of the total bound radioligand for probe ^{125}I-A, and probe ^{125}I-B, respectively. As compared to ^{125}I-rANP(1-28), ^{125}I-pBNP1, and [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27), for which values of 1%, 1%, and 2.5% are respectively obtained (4, 8) the addition of the N-terminal photophore causes a drastic effect on the hydrophobicity, especially in the case of [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]-pBNP1. It may suggest that proximity of Tyr^{2} with the benzophenone photophore produces a core with an enhanced hydrophobic character. High non-specific binding has rendered difficult the assessment of specific binding, a problem that has also been reported in the study of high affinity binding of pyrethroids to the alpha subunit of brain sodium channels (22). Computed incorporation efficiency percentages stand at 18% and 41% for probe ^{125}I-A, and probe ^{125}I-B, respectively. These yields are different to those of [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (70%) (4) or other reported benzophenone-modified ligands such as [Bpa^{8}]Substance P (70%) (23) or the myosine light-chain kinase analog [Bpa^{3}]MLCK (70%–90%) (24). Positioning of the benzophenone residue is critical in obtaining high incorporation yields. The photophore must be accessible to C-H bonds and it must spend sufficient time at the 0.3 nm interactive distance in order to make a covalent link (7). Bearing in mind the somewhat random coil

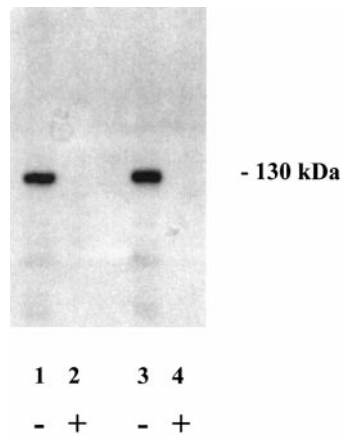

FIG. 5. NPR-A covalent photoaffinity labeling with ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]-pBNP1. Bovine adrenal zona glomerulosa membrane protein (20 μg/ml) was incubated with ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (20,000 cpm/ml) (lanes 1-2), or ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]-pBNP1 (20,000 cpm/ml) (lanes 3-4), in the absence (−) or presence (+) of rANP(1-28) (10^{-7} M) for 90 min at room temperature. Thereafter, the tubes were exposed to UV light 15 min and then centrifuged; the pellet was washed and resuspended in SDS-PAGE sample buffer. Each well was loaded with 40 μg of protein. Radioactive bands, detected by autoradiography, were cut and counted for radioactivity on a gamma-counter in order to determine incorporation.

nature of the N-terminal segment, these prerequisites may not be favored. Creation of a hydrophobic cluster near Arg^3- Arg^4 may also prove unfavorable. Nevertheless, our results still compare advantageously with typical incorporation efficiencies obtained with photoaffinity labeling experiments carried out with azido derivatives (≈1.0%) (25, 26) bifunctional reagents (5%–10%) (6), or underivatized ^{125}I-rANP(1-28) (1%–7%) (27).

By conducting these studies we have underline the synthesis feasibility of both photoprobes, and showed their high affinity. Both probes confirm previous results suggesting a participating role for ANP amino exocyclic segment in the ligand binding dynamic (3). Results also underline the feasibility of a bisubstituted pBNP1 photoaffinity labeling peptide. With optimization by proper tyrosine positioning for proteolytic fragment tracking, benzophenone adjunction at both ends of pBNP1 would make possible proteolytic fragmentation of each of the subunits of the NPR-A binding domain interacting with the probe, thus allowing further characterization of the binding environment.

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