Kishi, F., Maruyama, M., Tanizawa, Y., & Nakazawa, A. (1986) *J. Biol. Chem. 261*, 2942–2945.
Lewis, C. T., Haley, B. E., & Carlson, G. M. (1989) *Biochemistry 28*, 9248–9255.
McDonald, G. G., Cohn, M., & Noda, L. (1975) *J. Biol. Chem. 250*, 6947–6954.
Murakami, N., Healy-Louie, G., & Elzinga, M. (1990) *J. Biol. Chem. 265*, 1041–1047.
Muszynka, G., Andersson, L., & Porath, J. (1986) *Biochemistry 25*, 6850–6853.
Ng, K. E., & Orgel, L. E. (1987) *Nucleic Acids Res. 15*, 3573–3580.
Noda, L. (1973) *Enzymes (3rd Ed.) 8*, 279–305.
Owens, M. (1983) *Doctoral* Dissertation, University of Wyoming.
Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45.
Potter, R. L., & Haley, B. E. (1983) *Methods Enzymol. 91*, 613–633.
Reinstein, J., Schlichting, I., & Wittinghofer, A. (1990a) *Biochemistry 29*, 7451–7459.
Reinstein, J., Vetter, I. R., Schlichting, I., Rosch, P., Wittinghofer, A., & Goody, R. S. (1990b) *Biochemistry 29*, 7440–7450.
Salvucci, M. E., & Haley, B. E. (1990) *Planta 181*, 287–295.
Shyy, Y.-J., Tian, G., & Tsai, M.-D. (1987) *Biochemistry 87*, 6411–6415.
Smith, G. M., & Mildvan, A. S. (1982) *Biochemistry 27*, 6119–6123.
Tian, G., Sanders, C. R., Kishi, F., Nakazawa, A., & Tsai, M.-D. (1988) *Biochemistry 27*, 5544–5552.
Vetter, I. R., Reinstein, J., & Rosch, P. (1990) *Biochemistry 29*, 7459–7467.
Wente, S. R., & Schachman, H. W. (1991) *J. Biol. Chem. 266*, 20833–20839.
Yan, Y., Dahnke, T., Zhou, B., Nakazawa, A., & Tsai, M.-D. (1990) *Biochemistry 29*, 10956–10964.

Highly Efficient Photoaffinity Labeling of the Hormone Binding Domain of Atrial Natriuretic Factor Receptor†

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Received October 4, 1991; Revised Manuscript Received February 18, 1992

ABSTRACT: A high-efficiency photoaffinity derivative of atrial natriuretic factor (ANF) was developed for studying the peptide binding domain of the receptor protein and for better characterization of this receptor in tissues with a low density of binding sites. The position of the photosensitive residue was chosen on the basis of a molecular conformational model and on structure–activity relationship studies which both indicate that the carboxy-terminal end of the peptide is part of a hydrophobic pole likely to interact deeply within the ANF binding pocket of the receptor. Selection of the photoreactive residue *p*-benzoylphenylalanine (BPA) as a substitute for arginine in position 125 of the peptide sequence led to a photoaffinity derivative with a high (63%) efficiency of covalent incorporation to the receptor protein. This derivative (BPA-ANF) has a 10-fold lower affinity when compared with ANF, but it is a full agonist in stimulating cGMP production and inhibiting aldosterone secretion in bovine adrenal zona glomerulosa. Photoaffinity labeling with BPA-ANF specifically identifies ANF-R_1 and ANF-R_2 receptor proteins with a 10-fold higher efficiency than with azido derivatives of ANF or with cross-linking agents. This new ANF derivative therefore appears to be useful for studying ANF receptors in tissues with low levels of expression, for locating receptor following cellular internalization, and for tagging proteolytic fragments of the receptor amenable to amino acid microsequencing.

The discovery of the expanding family of cardiac natriuretic peptides and of their specific receptors has led to the identification of a unique type of transmembrane signaling mechanism for hormones and neuropeptides. These peptides, which include atrial natriuretic factor (ANF)[1] first identified in atrial extracts (Thibault et al., 1984; De Bold, 1985), brain natriuretic peptide (BNP) initially isolated from brain (Sudoh et al., 1988), and adrenal (Nguyen et al., 1989) and C-type

† This work was supported by grants from the Medical Research Council of Canada to the Research Program on Molecular Pharmacology and Genetics of Cardiac Natriuretic Peptides and for maintenance of the Molecular Modelling Resources (MT-10131) and from the Heart and Stroke Foundation of Quebec.

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[1] Abbreviations: ANF, atrial natriuretic factor; SDS, sodium dodecyl sulfate; IBMX, isobutylmethylxanthine; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid, sodium salt; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; BNP, brain natriuretic peptide; BPA-ANF, [Tyr[116],*p*-benzoyl-Phe[125]]ANF(99–125); C-ANF, [Cys[116]-ANF(102–116)-NH_2; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

0006-2960/92/0431-4487$03.00/0

natriuretic peptide (CNP) first localized in brain (Takei et al., 1990; Sudoh et al., 1990), all trigger their effect in target cells by stimulating a guanylate cyclase catalytic activity which is a constitutive part of their receptor (Chinkers & Garbers, 1991). Topographical studies (Liu et al., 1989) and molecular cloning (Chinkers et al., 1989; Lowe et al., 1989) of these receptors indicate that the 130-kDa glycoprotein involves a single transmembrane domain which separates the peptide chain in two nearly equal parts. The extracellular part includes the peptide binding domain which is less well conserved among the guanylate cyclase clones so far identified (Schultz et al., 1989). Despite those observations, little is known about the structural properties of the hormone binding domain of the ANF receptor.

Structure–activity relationships of atrial natriuretic factor have been extensively studied (Bovy, 1990). The results indicate the crucial role of the residues constituting the ring portion of the peptide (residues 105–121) (Schiller et al., 1986). The most essential segment includes residues Cys^{105}–Gly^{114}, and a corresponding linear decapeptide has been shown to be active as a vasorelaxant and natriuretic analogue (Bovy et al., 1989). The exocyclic carboxy-terminal residues are also important, especially Phe^{124} and Arg^{125}, while Tyr^{126} can be removed without losing biological potency (De Léan et al., 1985). The closely related natriuretic peptide BNP displays similar biological properties and shares significant homology with ANF both in the ring and in the carboxy-terminal portions (Nguyen et al., 1989). Conformational studies of these two peptides and especially of BNP suggest that the carboxy-terminal segment loops back toward the ring portion of the peptide (Inooka et al., 1990; Hobayashi et al., 1988), implying that they might be adjacent in the receptor-bound conformation of the peptide.

One method of characterizing the binding domain of peptide receptors involves the covalent attachment of radiolabeled derivatives either by photoactivation or with cross-linking agents. These approaches typically result in a low (≤1%) yield of covalent ligand–receptor complexes and usually involve attachment of the ligand through its amino terminus. In the case of natriuretic peptides, photosensitive azido derivatives have been used for identifying the ANF receptor but with a predictably low efficiency of covalent incorporation (Yip et al., 1985; Misono et al., 1985). Interpretation of the results of covalent incorporation of ANF photoaffinity derivatives following UV light treatment is complicated by the observation that even underivatized ANF can also be covalently attached apparently through a process involving membrane lipid peroxidation and generation of short-lived endogenous cross-linking agents (Larose et al., 1990). We therefore considered the need to develop a new high-yield photoaffinity derivative of ANF which should covalently attach to the binding domain through its more crucial carboxy terminus. The photoactivatable amino acid derivative *p*-benzoyl-L-phenylalanine (BPA) was selected because of its high (≤70%) efficiency of covalent incorporation (Kauer et al., 1986; Boyd et al., 1991) and was positioned at the carboxy terminus of the peptide. We report here the results obtained with this ANF derivative, which is 10 times more efficiently incorporated than other analogues reported so far.

Experimental Procedures

Materials. Rat ANF(99–126) was obtained from IAF Biochem, Montréal, Canada. [Cys^{116}]ANF(102–116)-NH_2 (C-ANF) was from Hukabel, Longueuil, Canada. Angiotensin II and CNP_{22} were from Peninsula. Bovine serum albumin, aprotinin, leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), collagenase, and DNase were from Sigma. Electrophoresis reagents and standards were from Bio-Rad. F-12 medium, horse serum, and fetal calf serum were from Gibco. Iodo-Beads were from Pierce Chemical Co., and carrier-free $Na^{125}I$ was from Amersham. ^{125}I-Labeled aldosterone, anti-aldosterone antibody, and goat anti-rabbit γ-globulin antiserum were obtained from Immunocorp, Montréal, Canada. cGMP was from Sigma, anti-cGMP was kindly provided by Dr. Alain Bélanger, Centre Hospitalier de l'Université Laval, Québec, Canada. ^{125}I-cGMP was prepared by radioiodination of 2′-*O*-monosuccinyl-cGMP tyrosyl methyl ester (Sigma), and the monoiodinated cGMP was purified on a reverse-phase Vydac C_{18} column. NIH-3T3 cells were obtained from American Type Culture Collection (Rockville, MD). GF/C filters were obtained from Fisher Scientific (Montréal, Canada). Poly(ethylenimine) was purchased from Aldrich (Milwaukee, WI).

Primary Cell Culture. Fresh bovine adrenal glands were obtained from a local slaughterhouse. The zona glomerulosa layer was dissected, dispersed with collagenase (2 mg/mL) and DNase (0.2 mg/mL), and plated (10^6 cells/mL) in F-12 medium, containing 10% horse serum, 2% fetal calf serum, and antibiotics, as previously described (De Léan et al., 1984). Cells were used after three resting days.

Preparation of Membranes. Bovine glomerulosa membranes were prepared according to the method of Meloche et al. (1988). Briefly, the zona glomerulosa layer was homogenized in buffer containing 20 mM $NaHCO_3$, 2 mM EDTA, 0.1 mM 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. The NIH-3T3 cells were grown as monolayers in Dulbecco's-modified Eagle's medium, supplemented with 5% fetal calf serum. As previously described (Fêthière et al., 1989), confluent NIH-3T3 cells were washed three times with 10 mL of saline and detached with a rubber policeman. They were homogenized in the $NaHCO_3$ buffer containing protease inhibitors and centrifuged. The pellet was washed twice, resuspended in buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.1 mM EDTA, and 1 mM $MgCl_2$, and frozen in liquid nitrogen.

Peptide Synthesis. The peptide [Tyr^{116},BPA^{125}]ANF(99–125) (BPA-ANF) was obtained by solid-phase synthesis. *p*-Benzoyl-L-phenylalanine (BPA) was first synthesized as previously described (Kauer et al., 1986) and then derivatized as FMOC-BPA. The synthesis was carried out at IAF Biochem (Montréal, Canada) by a BOP/HOBT stepwise solid-phase procedure on a Fmoc-X-Wang resin. The peptide was cleaved and deprotected with TFA and HF; then the cyclical form was obtained with I_2 and purified in two steps by HPLC on Vydac C_{18} columns. The peptide structure H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Tyr-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-BPA-OH was confirmed by gas-phase sequencing and amino acid analysis. The amino acid analysis yielded the following results [residue(s)/mol]: Asx = 2.00 (2), Gly = 4.63 (5), Ile = 1.60 (2), Ala = 0.97 (1), Leu = 1.76 (2), Ser = 4.25 (5), Cys = 1.05 (2), Tyr = 1.00 (1), Arg = 3.79 (4), and Phe = 1.84 (2). The amino acid sequence obtained was Ser-Leu-Arg-Arg-Ser-Ser-Xxx-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Tyr-Ser-Gly-Leu-Gly-Xxx-Asn-Ser-Phe-Xxx.

Iodination of BPA-ANF and ANF. BPA-ANF (7 nmol) or ANF was incubated with 1.5 mCi of $Na^{125}I$, in 140 μL of 0.25 M potassium phosphate buffer, pH 7.0, in the presence of two Iodo-Beads (Ong et al., 1987). The incubation was

performed at 4 °C for 20 min. The iodinated peptide was separated from the free iodine on a RP1 cartridge and purified on a reverse-phase Vydac C_{18} column. Elution was achieved at a flow rate of 1 mL/min with a 80-min linear gradient at 20–40% acetonitrile in 0.1% TFA. Fractions of 0.5 mL were collected and counted for radioactivity. The theoretical specific activity of the monoiodo derivative was 2000 Ci/mmol. All steps were done in the darkness.

Receptor Binding Assays. Membranes (20 μg) were incubated in the dark in 1 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.5% BSA, and 5 mM $MnCl_2$ for zona glomerulosa or 5 mM $MgCl_2$ for NIH-3T3 membranes, respectively, for 90 min at 22 °C. Bound radiolabeled ligand was determined by filtration on GF/C fiber glass filters, pretreated with 1% poly(ethylenimine), followed by washing with 50 mM potassium phosphate buffer (pH 7.4). Filters were counted in a LKB γ counter.

Guanylate Cyclase Activity. Guanylate cyclase activity was assayed according to the method of Garbers and Murad (1979). Briefly, adrenal zona glomerulosa membranes (10 μg) were incubated in 100 μL of buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM theophylline, 2 mM IBMX, 10 mM creatine phosphate, 10 units of creatine phosphokinase, and 1 mM GTP. After 10 min at 37 °C, 100 μL of 120 mM EDTA was added, and tubes were immersed in boiling water for 3 min. After addition of 1 mL of 50 mM ammonium acetate buffer, pH 7.0 the tubes were centrifuged, and cyclic GMP was quantified by radioimmunoassay after separation on an alumina column and derivatization by treatment with triethylamine/acetic anhydride (2:1).

Aldosterone Determination. Aldosterone measurement was done according to the modifications of Brochu et al. (1989). Incubation was carried out in 66 mM phosphate buffer, pH 7.0, containing 0.1% gelatin, 0.1% sodium azide, 0.005% normal rabbit serum, with antiserum (1:400 000), and ^{125}I-labeled aldosterone (10 000 cpm) at 4 °C overnight. A total of 500 μL of goat anti-rabbit γ-globulin antiserum diluted in 12% poly(ethylene glycol) was added, the tubes were centrifuged (supernatant removed), and the pellets were counted for radioactivity.

Photolabeling with [^{125}I]BPA-ANF. Radioligand binding was performed as described above. At the end of the incubation time, the tubes were purged with nitrogen and then placed on ice at a distance of 8 cm from two mercury lamps (54 mW cm^2 at 365 nM). After irradiation for the time specified, the tubes were centrifuged, and the pellets were washed twice with buffer containing 50 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, boiled for 3 min in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercaptoethanol, and 0.05% bromophenol blue), and subjected to 7.5% SDS–PAGE. Autoradiographic exposure was done at −80 °C with Kodak X-Omat XK-1 film with two intensifying screens.

Molecular Modeling of BPA-ANF. A model of ANF conformation was developed by computer modeling methods using the program SYBYL (Tripos, St. Louis, MO) running on a PS 330 graphics terminal (Evans & Sutherland) connected to a Vax station 3510 (Digital). The cyclic ANF peptide was first built and then energy minimized using NOE distance constraints obtained by NMR studies of the peptide in solution (Kobayashi et al., 1988). The model structure was subjected to 30 ps of dynamic simulation at 700 K in the absence of distance constraints in order to partially sample the allowed conformational space of this peptide. Conformers were sampled along the trajectory and minimized using the distance constraints; then the lowest energy conformer was selected. This structure of ANF was then modified to correspond to BPA-ANF and energy minimized in the absence of distance range contraints.

Data Analysis. Dose–response curves were analyzed by weighted least-squares regression using the computer program ALLFIT based on the four-parameter logistic equation (De Lêan et al., 1978). Receptor saturation curves were analyzed using the computer program SCAFIT based on a generalized form of the law of mass action (De Lêan et al., 1982).

Results

Molecular Modeling of BPA-ANF. Proper positioning of photoreactive amino acid residues requires prior knowledge of the structure of the native peptide and of its biologically important regions. In the case of ANF, both the intraloop segment Cys^{105}–Gly^{114} and the carboxy-terminal segment Phe^{124}–Arg^{125} appear to be essential for proper recognition of the peptide by the receptor and for its activation (Bovy, 1990). Molecular modeling of ANF and BNP conformation in solution based on NMR results also indicates that the carboxy-terminal segment of natriuretic peptides loops back toward the ring portion, thereby bringing close together these two biologically important regions (Inooka et al., 1990; Kobayashi et al., 1988). We assumed that the carboxy-terminal region was the best location for the photoreactive residue which replaced Arg^{125}. The radioiodination site Tyr^{126} was removed, and Gln^{116} was replaced by tyrosine, a substitution which does not appreciably affect biological activity (Goghari et al., 1990). A model for the conformation of BPA-ANF was then developed by modification of the molecular model for ANF (Figure 1). The three aromatic residues Tyr^{116}, Phe^{124}, and BPA^{125} all appear to be clustered together in the modeled structure forming a hydrophobic core. This spatial disposition can be expected to lead to a positioning of the photoreactive residue BPA deep within the peptide binding domain of the receptor. The BPA-substituted derivative of ANF was therefore selected for photoaffinity labeling studies.

Receptor Affinity and Biological Activity of BPA-ANF. The peptide [Tyr^{116},BPA^{125}]ANF(99–125) (BPA-ANF) was then synthesized and tested for biological activity. Figure 2 shows that BPA-ANF displays high affinity for both ANF-R_1 (ED_{50} = 490 pM, upper panel) and ANF-R_2 receptors (ED_{50} = 60 pM, lower panel). The photoreactive analogue is 13 times less potent than ANF(99–126), indicating that the modification and substitution of the peptide did not substantially alter its recognition by the receptor. The reduction in potency was even less pronounced (3-fold) in the case of the guanylate cyclase-independent receptor ANF-R_2.

The photoreactive analogue BPA-ANF is a full agonist. Figure 3 shows that BPA-ANF stimulates particulate guanylate cyclase with a relative potency of 0.12 (ED_{50} = 1.7 nM), in agreement with the results observed in receptor binding assays (Figure 2). The photoreactive analogue also inhibits aldosterone production (Figure 4; ED_{50} = 170 pM) and again behaves as a full agonist, when compared to ANF(99–126), with a relative potency of 0.05 which is commensurable with that observed for guanylate cyclase activity (0.12).

Covalent Photolabeling with [^{125}I]BPA-ANF. The results obtained demonstrate that BPA-ANF is a potent ANF analogue with full agonist activity. We therefore prepared and tested the radiolabeled form of the analogue. Purification of [^{125}I]monoiodo-BPA-ANF yielded a radioligand with a specific activity around 2000 Ci/mmol. The radiolabeled peptide eluted on a C_{18} reverse-phase column at a 5% higher concentration of acetonitrile than [^{125}I]ANF(99–126), indicating that BPA-ANF behaves as a slightly more hydrophobic peptide

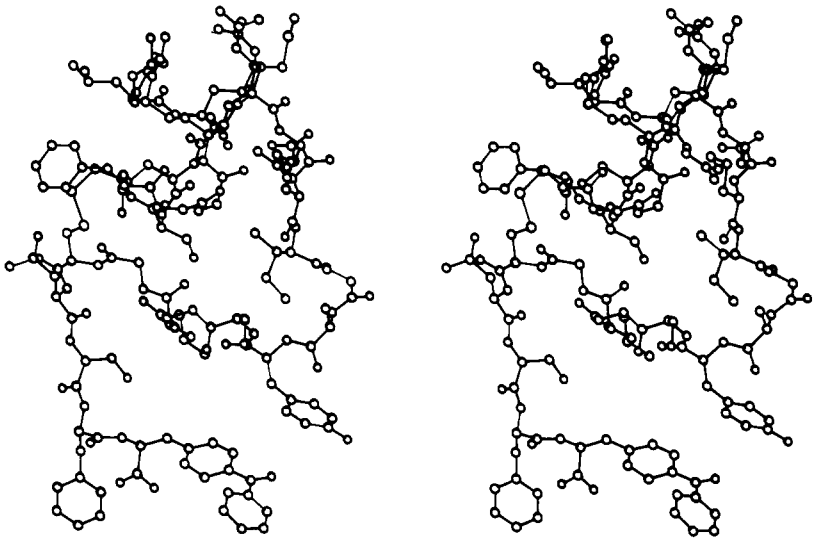

FIGURE 1: Stereoscopic model of BPA-ANF. A molecular model of ANF was derived by molecular modeling using interprotein distance constraints obtained by NMR studies of the peptide. The model was then modified by substitution and deletion in order to obtain [Tyr[116],BPA[125]]ANF(99–125) and again energy minimized but in the absence of distance constraints. Hydrogen atoms were omitted for clarity.

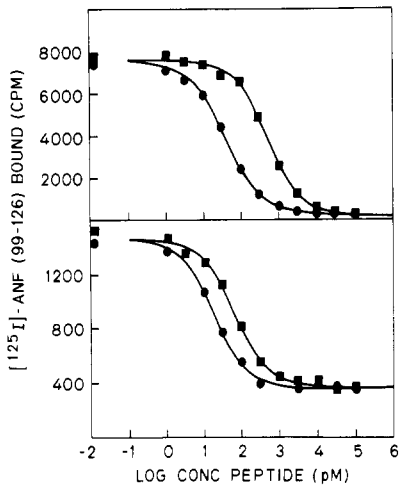

FIGURE 2: Relative potency of BPA-ANF in competitive binding in ANF-R_1 and ANF-R_2 receptors. Adrenal zona glomerulosa membranes (20 μg) containing ANF-R_1 receptors (upper panel) or NIH-3T3 membranes (20 μg) containing ANF-R_2 receptors (lower panel) were incubated with 30 000 cpm of [^{125}I]ANF(99–126) and varying concentrations of ANF(99–126) (●) or BPA-ANF (■). Total radioligand binding measured after separation and washing on GF/C filters is reported. Curves were analyzed by the computer program (De Lêan et al., 1978) for nonlinear least-squares regression.

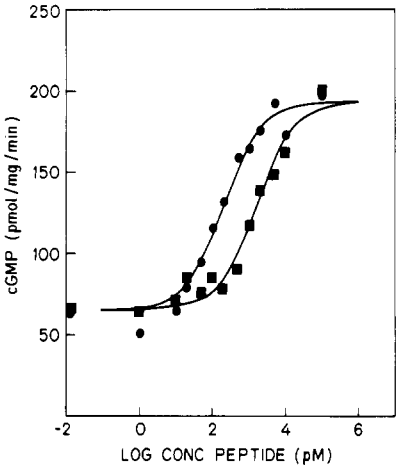

FIGURE 3: Relative potency of BPA-ANF in stimulating guanylate cyclase in zona glomerulosa membranes. Bovine adrenal zona glomerulosa membranes (10 μg) were incubated with 1 mM GTP, 4 mM $MnCl_2$, and varying concentrations of ANF(99–126) (●) or BPA-ANF (■). Cyclic GMP was isolated on a neutral alumina column and measured by radioimmunoassay (Garbers & Murad, 1979). Dose–response curves were analyzed using the computer program ALLFIT.

than the parent compound. The radiolabeled peptide reversibly and competitively binds ANF receptor sites in the absence of photoreaction. Figure 5 shows results similar to those obtained with [^{125}I]ANF(99–126) (Figure 2) except that the photoreactive analogue displayed significantly higher nonspecific binding. This nonspecific interaction might be related to the higher hydrophobicity of the analogue.

The photoreactive analogue was then covalently incorporated into the ANF receptor binding domain by UV activation at 365 nm. The activation was carried out in the absence of oxygen in order to minimize spontaneous covalent incorporation of ANF due to lipid peroxidation (Larose et al., 1990). The time course of photoaffinity labeling obtained at 130 pM [^{125}I]BPA-ANF was documented by autoradiography following SDS–PAGE (Figure 6). The results indicate that covalent incorporation of BPA-ANF reaches a steady-state level after 20 min of UV exposure. The high yield of covalent incorporation (3000 cpm/20 μg of membrane) corresponds to 50–60% of the reversibly bound radioligand amounting to 20% of the total receptor binding capacity. The yield of maximum covalent attachment of BPA-ANF to the receptor binding sites was then measured by using a saturating concentration of [^{125}I]BPA-ANF (Figure 7). The results indicate that 64% of the total receptor binding sites (0.578 pmol/mg) were covalently modified. This high yield is in agreement with those obtained with other BPA-substituted ligands (Kauer et al., 1986; Boyd et al., 1991).

Covalent incorporation of BPA-ANF results in irreversible loss of ANF receptor binding capacity. BPA-ANF was incorporated by photoactivation followed by washing with 10 mM DTT, in order to remove noncovalently bound ligand. [^{125}I]ANF(99–126) binding was irreversibly abolished following incorporation of BPA–ANF at a saturating concentration (10 nM) (data not shown). The [^{125}I]ANF binding capacity was recovered if UV exposure was omitted.

Finally, we tested the suitability of BPA–ANF to photolabel proteolytic fragments of interest for amino acid microsequencing of the receptor. Figure 8 shows that the BPA-ANF photoaffinity-labeled ANF receptor yields a radiolabeled

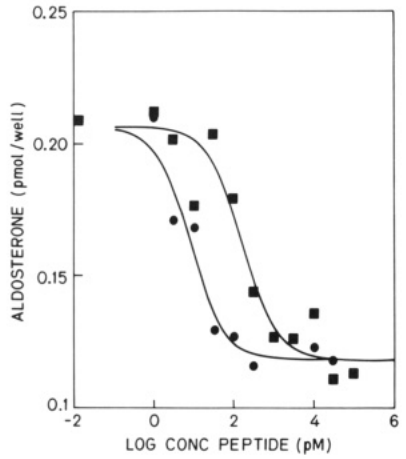

FIGURE 4: Relative potency of BPA-ANF in inhibiting angiotensin II stimulated aldosterone secretion in adrenal zona glomerulosa cells. Bovine adrenal zona glomerulosa cells (10^6 cells/well) in primary culture were incubated for 3 h with 10 nM angiotensin II and increasing concentrations of ANF(99–126) (●) or BPA-ANF (■). Aldosterone production accumulating in the culture medium was measured by radioimmunoassay. Curves were simultaneously analyzed by least-squares regression using a four-parameter logistic model.

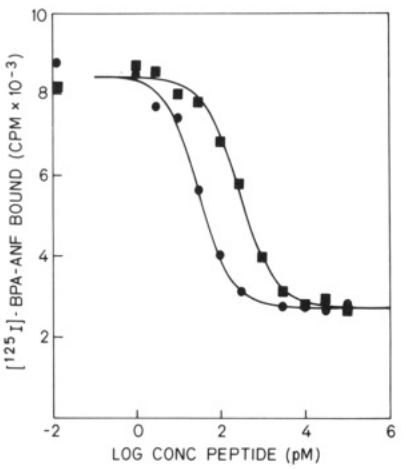

FIGURE 5: Specificity of [^{125}I]BPA-ANF competitive binding to adrenal zona glomerulosa membranes. Bovine adrenal zona glomerulosa membranes (20 μg) were incubated in the dark with [^{125}I]-BPA-ANF (100 000 cpm) and varying concentrations of ANF(99–126) (●) or BPA-ANF (■). Results are reported as total radioligand binding (specific and nonspecific). Curves were analyzed using the computer program ALLFIT.

fragment when proteolyzed with trypsin but not with chymotrypsin. These results are in agreement with the lack of a trypsin cleavage site between Tyr^{116} and BPA^{125} but with the presence of a chymotrypsin cleavage site after Phe^{124} in the BPA-ANF sequence. It is therefore suitable to use BPA-ANF for tagging a tryptic fragment of the ANF receptor binding domain.

Discussion

We have shown that the photoreactive analogue [Tyr^{116},BPA^{125}]ANF(99–125) is a very efficient compound for photoaffinity labeling of the ANF receptor. The high (69–70%) efficiency of covalent incorporation of this compound is at least 10 times better than that reported for affinity cross-linking with ANF (0.6%; Larose et al., 1990) or photoaffinity labeling with azido derivatives (0.5–1%; Pandey et al., 1986), and therefore affinity labeling with this compound is extremely useful in drastically reducing both the amount of protein loaded for SDS–PAGE and the time of exposure

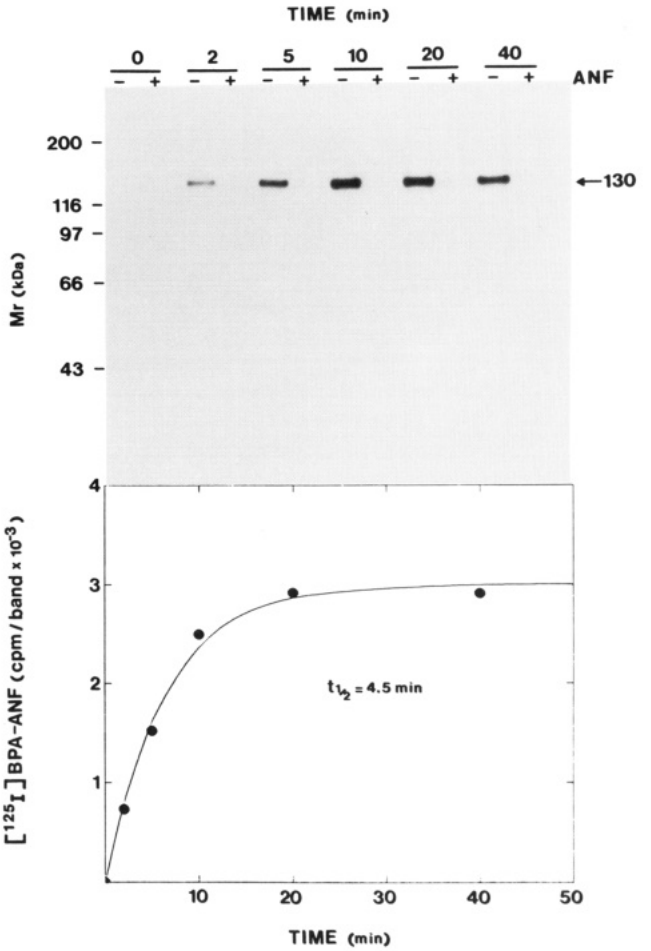

FIGURE 6: Time course of covalent photolabeling with [^{125}I]BPA-ANF. Bovine adrenal zona glomerulosa membranes (400 μg/5 mL) were incubated with [^{125}I]BPA-ANF (2×10^6 cpm/5 mL) in the absence (−) or the presence (+) of 100 nM ANF(99–126) for 90 min at 22 °C. Tubes were then exposed to UV light, and 0.5-mL (40-μg) aliquots were washed and resuspended in SDS–PAGE sample buffer. Each lane was loaded with 20 μg of protein. Radioactive bands detected by autoradiography (upper panel) were cut and measured in a γ counter (lower panel).

of gels for autoradiography. Thus it is practical to use BPA-ANF for autoradiographic detection of ANF receptors even in tissues with a low density of binding sites and for which previously established methods yielded faint bands and poor quantitative results. The advantage of using benzophenone derivatives of ANF for photoaffinity labeling of the receptor is in agreement with those reported in earlier studies (Kauer et al., 1986; Boyd et al., 1991). The ability of the photoexcited state of the derivative to return to the ground state if no covalent incorporation has occurred is apparently crucial to its high efficiency when compared with azido derivatives (Kauer et al., 1986). Modification of ANF to yield BPA-ANF resulted in only a 10-fold reduction in affinity but full maintenance of its agonist activity. This acceptable reduction in affinity is easily compensated by using higher concentrations of the radiolabeled derivative. The increased hydrophobicity of BPA-ANF due to the positioning of Tyr^{116}, Phe^{124}, and BPA^{125} at a pole of the molecule (Figure 1) presumably favors its covalent attachment well within the ANF binding pocket of the receptor. It does, however, also result in significantly higher nonspecific binding (2.8% of total ligand added). Proper removal and washing of unbound radioligand are required to maintain a low background for autoradiographic detection of the receptor.

The molecular model for ANF developed from NMR

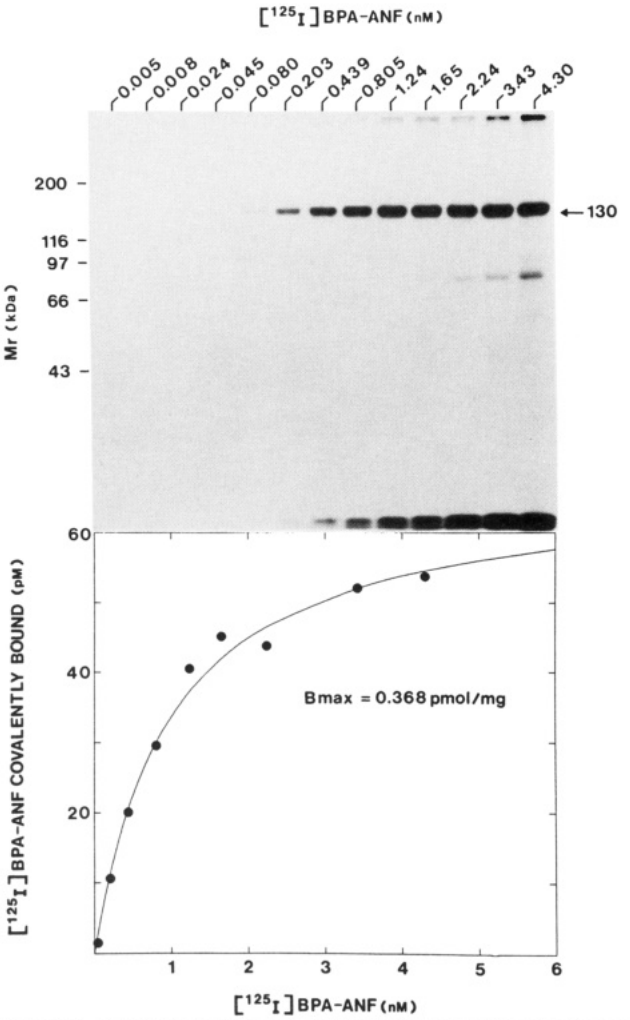

FIGURE 7: Covalent receptor saturation with UV photoactivation of [^{125}I]BPA-ANF. [^{125}I]BPA-ANF (quenched to 122 Ci/mmol) was incubated with bovine adrenal zona glomerulosa membranes (200 μg in 1 mL of binding buffer) and then UV photoactivated. The radioligand–receptor covalent complex was washed and resuspended in SDS–PAGE sample buffer. Each lane was loaded with 40 μg of protein. The radioligand covalently incorporated was evaluated by autoradiography (upper panel), and the radioactive bands were cut and counted (lower panel). The resulting saturation curve was analyzed using SCAFIT, a computer program based on the law of mass action.

studies of its conformation in solution predicts that BPA–ANF would be an efficient photoaffinity derivative because of the positioning of the photoreactive residue BPA within the carboxy-terminal region, which is essential for potent biological activity of the peptide. The results obtained with BPA–ANF are in agreement with the model. An additional property of BPA-ANF to covalently incorporate a radiolabeled fragment which is still present after trypsin cleavage provides a new approach for studying the ANF binding domain of the receptor. Similar approaches have been successfully applied to the study of the EGF receptor which also includes a large extracellular EGF binding domain (Wu et al., 1990). Preliminary results indicate that the use of BPA–ANF for probing and identifying the site of its covalent attachment is advantageous for delineating which section of the extracellular domain is intimately associated with binding of the peptide. These results together with proper molecular studies of the identified region by site-directed mutagenesis should contribute to a better understanding of the ANF recognition function of its receptor and of the structural properties of its binding domain.

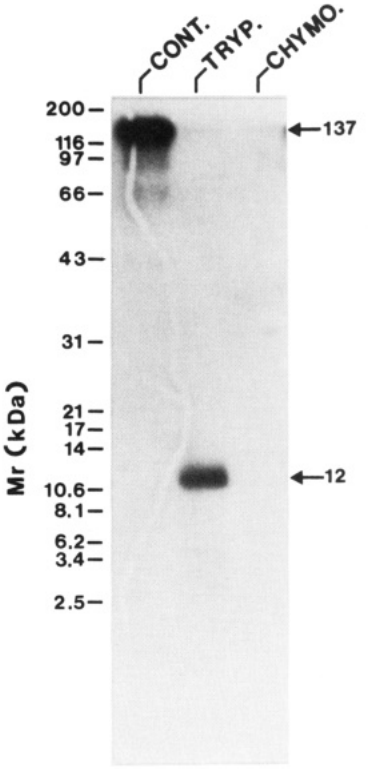

FIGURE 8: Proteolytic cleavage of the [^{125}I]BPA-ANF–receptor covalent complex. Bovine adrenal zona glomerulosa membranes (200 μg) were incubated with 750 000 cpm of [^{125}I]BPA-ANF, followed by UV photoactivation. The covalently labeled membrane receptor was solubilized and isolated in 7.5% SDS–PAGE. The radioactive band was eluted and then incubated with either buffer alone (left lane), trypsin (center lane), or chymotrypsin (right lane). The proteolysis reaction medium was again sampled on 10% SDS–PAGE using Tricine as the trailing ion (Schägger et al., 1987) and analyzed by autoradiography.

ACKNOWLEDGMENTS

We thank Dr. Paulo Bouça from IAF Biochem International (Montréal) for his collaborative effort in synthesizing the photoaffinity derivative of ANF and Mrs. Isabelle Blain for preparing the manuscript.

Registry No. [Tyr^{116},BPA^{125}]ANF(99–125), 139915-02-9; ANF, 85637-73-6; cGMP, 7665-99-8; aldosterone, 52-39-1.

REFERENCES

Bovy, P. R. (1990) *Med. Res. Rev. 10*, 115–142.

Bovy, P. R., O'Neal, J. M., Olins, G. M., Patton, D. R., Mehta, P. P., McNahon, E. G., Palomo, M., Schuh, J., & Blehm, D. (1989) *J. Biol. Chem. 264*, 20309–20313.

Boyd, N. D., White, C. F., Cerpa, R., Kaiser, E. T., & Leeman, S. E. (1991) *Biochemistry 30*, 336–342.

Brochu, M., Fêthière, J., Roy, M., Ong, H., & De Lêan, A. (1989) *Clin. Biochem. 22*, 289–292.

Chinkers, M., & Garbers, D. L. (1989) *Science 245*, 1392–1394.

Chinkers, M., & Garbers, D. L. (1991) *Annu. Rev. Biochem. 60*, 553–575.

Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H., Goeddel, D. V., & Schultz, S. (1989) *Nature 338*, 78–83.

De Bold, A. J. (1985) *Science 230*, 767–770.

De Lêan, A., Munson, P. J., & Rodbard, D. (1978) *Am. J. Physiol. 235*, E97–E102.

De Lêan, A., Hancock, A. A., & Lefkowitz, R. J. (1982) *Mol. Pharmacol. 21*, 5–16.

De Lêan, A., Racz, K., Gutkowska, J., Nguyen, T. T., Cantin, M., & Genest, J. (1984) *Endocrinology 115*, 1636–1638.
De Lêan, A., Thibault, G., Seidah, N. G., Lazure, C., Gutkowska, J., Chrêtien, M., Genest, J., & Cantin, J. (1985) *Biochem. Biophys. Res. Commun. 132*, 360–367.
Fêthière, J., Meloche, S., Nguyen, T. T., Ong, H., & De Lêan, A. (1989) *Mol. Pharmacol. 35*, 584–592.
Garbers, D. L., & Murad, F. (1979) *Adv. Cyclic Nucleotide Res. 10*, 57–67.
Goghari, M. H., De Lêan, A., Garcia, R., Cantin, M., & Schiller, P. W. (1990) *Int. J. Pept. Protein Res. 36*, 156–160.
Inooka, H., Kikuchi, T., Endo, S., Ishibashi, Y., Wakimasu, M., & Nizuta, E. (1990) *Eur. J. Biochem. 193*, 127–134.
Kauer, J. C., Erichser-Viitaner, S., Wolfe, H. P., Jr., & De Grado, W. (1986) *J. Biol. Chem. 261*, 10695–10700.
Kobayashi, Y., Ohkubo, T., Kyojoku, Y., Koyama, S., Kobayashi, M., & Go, N. (1988) *J. Biochem. 104*, 322–325.
Larose, L., McNicoll, N., Rondeau, J. J., Escher, E., & De Lêan, A. (1990) *Biochem. J. 267*, 379–384.
Liu, B., Meloche, S., McNicoll, N., Lord, C., & De Lêan, A. (1989) *Biochemistry 28*, 5599–5605.
Lowe, D. G., Chang, M. S., Hellmiss, R., Chen, E., Singh, S., Garbers, D. L., & Goeddel, D. V. (1989) *EMBO J. 8*, 1377–1384.
Meloche, S., McNicoll, N., Liu, B., Ong, H., & De Lêan, A. (1988) *Biochemistry 27*, 8151–8158.
Misono, K. S., Grammer, R. T., Rigby, J. W., & Inagami, T. (1985) *Biochem. Biophys. Res. Commun. 130*, 994–1001.
Nguyen, T. T., Lazure, C., Babinski, K., Chrêtien, M., Ong, H., & De Lêan, A. (1989) *Endocrinology 124*, 1591–1593.
Ong, H., Meloche, S., De Lêan, A., & Larose, P. (1987) *J. Lipid Chromatogr. 10*, 3085–3100.
Pandey, K. N., Inagami, T., & Misono, K. S. (1986) *Biochemistry 25*, 8467–8472.
Schägger, H., & Von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Schiller, P. W., Bellini, F., Dionne, G., Maziak, L. A., Garcia, R., De Lêan, A., & Cantin, M. (1986) *Biochem. Biophys. Res. Commun. 138*, 880–885.
Schultz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., & Garbers, D. L. (1989) *Cell 58*, 1155–1162.
Sudoh, T., Kangawa, K., Minamino, N., & Matsuo, H. (1988) *Nature 332*, 78–81.
Takei, Y., Takanashi, A., Watanabe, T. X., Takajima, K., Sakakibara, S., Takao, T., & Shimonishi, Y. (1990) *Biochem. Biophys. Res. Commun. 170*, 883–891.
Thibault, G., Garcia, R., Cantin, M., Genest, J., Lazure, C., Seidah, N. G., & Chrêtien, M. (1984) *FEBS Lett. 167*, 352–356.
Wu, D., Wang, L., Chi, Y., Sato, G. H., & Sato, J. D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3151–3155.
Yip, C. C., Laing, L. P., & Flynn, T. G. (1985) *J. Biol. Chem. 260*, 8229–8232.

Signal Transmission by Epidermal Growth Factor Receptor: Coincidence of Activation and Dimerization†

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Received August 7, 1991; Revised Manuscript Received December 30, 1991

ABSTRACT: Dimerization of epidermal growth factor receptor dissolved in a solution of nonionic detergent was followed with a resolution of 1 min by quantitative cross-linking with glutaraldehyde. Upon addition of epidermal growth factor to the solution, the initially monomeric protein dimerized in a reaction that was second-order in the concentration of receptor. A second-order rate constant, on the basis of enzymatic activity as a measure of the concentration of functional receptor, was calculated from time courses of dimerization at various initial concentrations of receptor. The activation of the protein tyrosine kinase of the receptor was monitored directly under the same conditions with an exogenous substrate. The increase in tyrosine kinase activity displayed kinetics that were also second-order in the concentration of receptor. A second-order rate constant for the activation of the tyrosine kinase could be calculated from the time courses. The second-order rate constant for the activation of the tyrosine kinase by epidermal growth factor was indistinguishable from the second-order rate constant for the dimerization induced by epidermal growth factor. Therefore, dimerization of epidermal growth factor receptor and activation of its tyrosine kinase are coincident events, both initiated by the binding of epidermal growth factor.

Epidermal growth factor receptor (EGFR)[1] is a transmembrane glycoprotein composed from a folded polypeptide 1190 amino acids in length (Ullrich et al., 1984). It is a member of a group of structurally and functionally related cell surface receptors characterized by possessing an intrinsic activity for protein tyrosine kinase (Yarden & Ullrich, 1988; Gill et al., 1987). It comprises an extracellular domain where the binding site for epidermal growth factor (EGF)[1] resides and a cytoplasmic domain where the active site for the tyrosine kinase is located. On the basis of the amino acid sequence of the protein, it has been proposed that a single membrane-

† This research was supported by Grant GM33962 from the National Institutes of Health that provides funds for the laboratory of Jack Kyte and by a postdoctoral fellowship from the Spanish Ministerio de Educacion y Ciencia.

[1] Abbreviations: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PVDF, poly(vinylidene difluoride); SDS-PAGE, electrophoresis on a polyacrylamide gel cast in a solution of sodium dodecyl sulfate.

0006-2960/92/0431-4493$03.00/0