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DÉVELOPPEMENT DE SONDES PHOTOSENSIBLES POUR LE MARQUAGE RÉGIO-SPÉCIFIQUE DU RÉCEPTEUR NPR-A BOVIN

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Résumé

ans le but de mieux cerner la dynamique des interactions existant entre les peptides Dnatriurétiques et le récepteur natriurétique de type-A, nous avons procédé à des études systhématiques de photomarquage de haute affinité avec le benzophénone comme agent photoactivable, et l'analogue chimérique de haute affinité [N,C,rANP(1-28)]pBNP32 (identifié comme le pBNP1) (Mimeault et al., 1993, *Mol. Pharmacol. 43*, 775-782), comme gabarit de synthèse. Au total, huit sondes ont été synthétisées sur support solide par chimie Boc/BOP. Les sondes [N^{α}-*p*-benzoylbenzoyl, L2Y]pBNP1 (A), ainsi que [N^{α}-*p*-benzoylbenzoyl, L18Y]pBNP1 (B), ont été développées de façon à incorporer l'agent benzophénone directement en N^{α}, avec pour objectif d'étudier une interaction possible entre le segment exocyclique situé en amino-terminal de l'ANP, et le site de reconnaissance du récepteur NPR-A. Dans les deux cas, le BOP a été utilisé comme agent de couplage de l'acide *p*-benzoylbenzoïque pour la dérivation en N^{α}. Les sondes [L2Y, R3Bpa]pBNP1 (C), [S6Y, G9Bpa]pBNP1 (D), et [G16Bpa, L18Y]pBNP1 (E), ont été synthétisées de façon à incorporer l'agent benzophénone à l'intérieur du cycle de l'ANP, tandis que les sondes [L18Bpa, F26Y]pBNP1 (F), [L18Y, F26Bpa]pBNP1 (G), et [L18Y, Y28Bpa]pBNP1 (H), ont été développées de façon à incorporer l'agent benzophénone dans le segment exocyclique situé en carboxy-terminal. Dans tous les cas, l'agent photosensible a été incorporé via l'utilisation de l'acide aminé modifié *p*-benzoyl-L-phénylalanine. Les potentiels de liaison ont été évalués sur des préparations membranaires de cortex de glande surrénale de boeuf. Sous leur forme linéaire, tous les peptides ont démontré une faible affinité. À l'inverse, les analogues cycliques pBNP1-A, -B, -C, -F, -G, et -H ont enregistré des affinités statistiquement équivalentes à celle démontrée par l'ANP (Δ pK_i : de -0.04 à +0.14), alors que pBNP1-D (Δ pK_i = -0.43 ± 0.10) a enregistré une affinité 3 fois moindre. Considérant sa faible affinité, pBNP1-E (Δ pK_i = -1.76 ± 0.08) a été rejeté comme sonde potentielle. L'iodation des analogues via l'utilisation de la lactoperoxydase s'est caractérisée par l'iodation préférentielle du résidu Tyr^{28}. De l'incorporation du benzophénone au niveau du segment N-terminal (pBNP1-A, -B, -C) ont découlé les sondes affichant les plus importants taux de liaison non-

spécifique, situés à 34%, 22%, et 20%, respectivement, alors que des substitutions faites à l'intérieur du cycle (pBNP1-E, -F), et dans le segment exocyclique C-terminal (pBNP1-G, -H), ont decoulé des sondes affichant des taux de liaison non-spécifique situés à 15%, 14%, 7%, et 6%, respectivement. Les sondes ^{125}I-pBNP1-A, -B, -C, -F, -G, et -H ont toutes marqué de façon spécifique le récepteur NPR-A monomérique (130 kDa). Au contraire, ^{125}I-pBNP1-E s'est montré incapable de toute incorporation. Les taux maximum d'incorporation ont été mesurés à 18%, 41%, 6%, 25%, 40%, et 40%, pour ^{125}I-pBNP1-A, -B, -C, -F, -G, et -H, respectivement. La digestion du complexe obtenu par le photomarquage du récepteur avec chacune des sondes a révélé deux motifs distincts en SDS-PAGE, avec ^{125}I-pBNP1-A, -B, -C, -G, et -H, affichant une bande majeure à 16 kDa, et des bandes additionnelles à 40, 34, 13, et 4 kDa, alors que le motif obtenu avec pBNP1-F n'a affiché que deux bandes, une majeure à 4 kDa, et une mineure à 40 kDa. La réduction et l'alkylation de ce fragment de 4 kDa, et son analyse subséquente par 16T6C SDS-PAGE a révélé une bande de poids moléculaire supérieure à celle obtenue par la migration de la sonde réduite et alkylée, mais non complexée au récepteur. Ces résultats suggèrent la présence d'un nouveau point de contact entre les peptides natriurétiques et le récepteur NPR-A.

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Table des matières

Première Partie

1. Cinquante ans déjà.

En regard de la haute incidence des maladies cardio-vasculaires touchant les Nord-américains, et compte tenu de l'importance que jouent les peptides natriurétiques dans le maintien de l'homéostasie cardio-vasculaire, il était normal que l'identification de ces facteurs hormonaux, réalisée dans la seconde moitié des années 80 concoure à la naissance d'un nouveau champ de recherche intense. Si l'identification d'un premier facteur natriurétique fut reçu par nombre de laboratoires comme le signal de départ vers un nouvel axe d'activité, il n'en demeure pas moins que cette découverte mettait également fin à une quête amorcée près d'un demi-siècle auparavant. C'est dès 1935 que fut proposé le concept impliquant le volume sanguin comme effecteur natriurétique (cité dans McDowell et al., 1995). En 1955 fut observée la présence de granules dans les cardiocytes auriculaires (Kisch, 1955). L'année suivante on s'aperçut que la distension mécanique de l'oreillette droite provoquait une diurèse chez le chien (Henry et al., 1956), et en 1976, leurs nombre et densité furent corrélés avec des déséquilibres électrolytiques (Maria et al., 1976). Enfin, 1981 voyait l'utilisation d'extraits d'oreillette de coeur de rat dans l'induction d'une diurèse chez le même animal (De Bold et al., 1981). Le premier facteur natriurétique était découvert, et fut baptisé ANP (*atrial natriuretic peptide*).

La qualité de notre expertise scientifique, appuyée bien sûr par le support financier d'organismes subventionnaires, permit à plusieurs chercheurs québécois de participer dès le début à l'effort collectif de recherche. Le rôle de l'IRCM sera prépondérant, avec la présence de groupes hautement qualifiés, tels ceux des docteurs Genest et Cantin qui à eux deux cumuleront au cours de leur carrière plus de 200 publications concernant directement les peptides natriurétiques, ainsi que ceux des docteurs Chrétien et De Léan. Ce dernier (co-directeur du présent mémoire), demeure d'ailleurs un leader incontesté de la recherche sur les peptides natriurétiques, avec la publication, au cours des dix dernières années, de plus de cinquante articles scientifiques dans le domaine. Ainsi, quinze années après la découverte du premier facteur natriurétique, la communauté scientifique dispose-t-elle d'une imposante somme de renseignements colligés dans plus de 5000 publications (Fig. 1). À ce jour, les peptides natriurétiques ont été localisés dans plusieurs tissus de mammifères et d'invertébrés (Hagiwara et al., 1995). Ils ont été purifiés et ont

fait l'objet d'une caractérisation pharmacologique poussée (Lewicki et Protter, 1995). Leur structure tertiaire a été modélisée par analyses de résonance magnétique nucléaire en présence de dymethyl sulfoxyde ou encore de phospholipides (Fairbrother et al., 1994; Carpenter et al., 1997). Sans doute parce que premier à être découvert, l'ANP, comme le démontre la figure 1, demeure

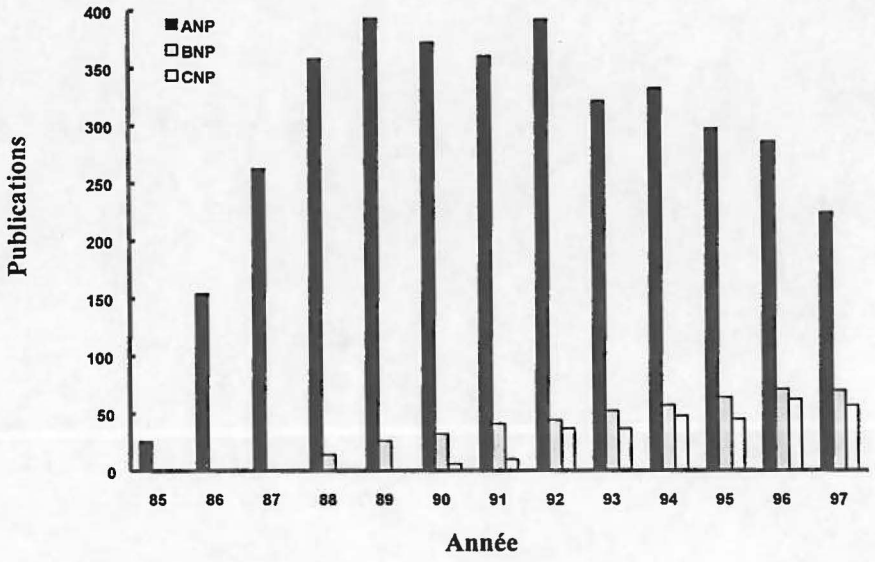


Figure 1. Nombre d'articles scientifiques publiés en fonction des années, et concernant directement les domaines rattachés à l'étude des peptides natriurétiques. (Selon une analyse non-exhaustive de la banque de données Medline).

de loin le facteur le mieux connu, et comme tel, il a fait l'objet d'analyses de structure-activité détaillées (Bovy, 1990) ainsi que d'études de minimisation structurale (Li et al., 1995). Trois récepteurs, localisés par autoradiographie ou par analyses immunohistochimiques dans les tissus cibles ont été identifiés, purifiés et clonés chez plusieurs espèces (Hagiwara et al., 1995; Yuen et Garbers, 1992 *pour des revues*). Ils ont fait l'objet d'analyses de structure\activité par mutagénèse dirigée (Duda et al., 1991; Iwashina et al., 1993; Itakura et al., 1997); et par expression de clones tronqués (Chinkers et Wilson, 1992). Les effets physiologiques, médiés par les récepteurs natriurétiques, ont fait l'objet d'études extensives (Anand-Srivastava et al., 1993; Chinkers et Garbers, 1991).

2. Peptides natriurétiques: Contexte actuel.

2.1. Homologie Structurale

L'ANP (*atrial natriuretic peptide*), initiallement isolé de cardiocytes auriculaires de coeurs humain et de rat (Thibault et al., 1984; De Bold, 1981), le BNP *(brain natriuretic peptide*), initiallement identifié dans des tissus de cerveau de porc (Sudoh et al., 1988), ainsi que le CNP (*C-Type natriuretic peptide*), également isolé en tout premier lieu d'extraits de cerveau de porc (Sudoh et al., 1990), constituent les membres actuels de la famille des peptides natriurétiques.

hANP	**SLRRSSCFGGRMDRIGAQSGLGCNSFRY**
hBNP	**SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH**
hCNP	**GLSKGCFGLKLDRIGSMSGLGC**

Figure 2. Séquence primaire des membres de la famille des peptides natriurétiques chez l'humain (h). Les acides aminés conservés sont soulignés. Les résidus cystéines (C) sont impliqués dans un pont disulfure intramoléculaire.

ANP	**humain, porc**	**SLRRSSCFGGRMDRIGAQSGLGCNSFRY**
	anguille	**SKSSSPCFGGKLDRIGSYSGLGCNSRK**
BNP	**humain**	**SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH**
	volaille	**MMRDSGCFGRRIDRIGSLSGMGCNGSRRKN**
CNP	**humain, rat**	**GLSKGCFGLKLDRIGSMSGLGC**
	salamandre	**GQSSGCFGLKLDRIGSMSGLGC**
	requin	**GPSRSCFGLKLDRIGAMSGLGC**

Figure 3. Homologie entre peptides natriurétiques, tous sous-types confondus, issus de mammifères, d'invertébrés et de poissons. Les résidus conservés sont représentés en rouge.

Outre des effets physiologiques similaires, et comme le souligne la figure 2, le regroupement des peptides natriurétiques tient également à une homologie de structure, caractérisée par la présence d'un cycle de 17 acides aminés fermé par un pont disulfure et à l'intérieur duquel 11 résidus sont conservés. Une analyse phylogénique plus étendue permet de souligner la haute homologie de structure reliant les peptides natriurétiques à travers tout le règne animal (Fig. 3). En effet, outre la conservation du cycle de 17 acides aminés, on remarque la conservation de 9 résidus, nonobstant l'importante distance phylogénique séparant les poissons des mammifères

2.2. Tissus cibles et effets physiologiques

L'ANP et le BNP sont appelés à intervenir pour palier toute surcharge hémodynamique et ainsi assurer l'homéostasie cardio-vasculaire. Selon un mécanisme encore mal connu, et initié par une distension de l'oreillette droite causée par une surpression, ces deux facteurs hormonaux, stockés sous forme de granules dans les cardiocytes auriculaires, sont libérés sous forme prohormonale et clivés enzymatiquement en leur forme active lors de l'exocytose (Lewicki et Protter, 1995). Plusieurs tissus ciblés par l'ANP et le BNP ont été identifiés. Des sites spécifiques de liaison ont été principalement localisés au niveau des reins, des glandes surrénales, et du muscle vasculaire lisse (Mantyh et al., 1986; Maeda et al., 1990; Wilcox et al., 1991). La liaison à ces sites de l'ANP et du BNP se traduit respectivement par une filtration glomérulaire accrue associée à une natriurèse et une diurèse (De Bold et al., 1981; Cantin et al., 1985; Sudoh et al., 1988), une inhibition de la synthèse d'aldostérone (De Léan et al., 1984), et une perméabilité accrue du lit vasculaire (Currie et al., 1983; Sudoh et al., 1988). ANP et BNP antagonisent également l'effet d'hormones telles l'endothéline, la vasopressine ainsi que certains minéralocorticoïdes. Le CNP demeure de tous les facteurs natriurétiques le moins étudié. Mentionnons qu'il est un puissant veinodilatateur (Stingo et al., 1992).

3. Transduction du signal: cGMP comme second messager

La transduction de signaux hormonaux via l'intermédiaire de récepteurs transmembranaires joue un rôle prépondérant dans la signalisation cellulaire (Waldman et Murad, 1987). Le nucléotide cyclique 3', 5' guanosine monophosphate (cGMP) intervient dans plusieurs cas comme second messager. Un accroissement de la concentration cytoplasmique du nucléotide module l'activité de protéines kinases cGMP-dépendantes (Corbin et al., 1990), de phosphodiestérases cGMP-dépendantes (Beavo, 1988) ou encore de canaux ioniques sensibles à l'action du cGMP (Fesenko et al., 1985; Yau et al., 1989). Deux isoformes de l'enzyme guanylyl cyclase (GTP pyrophosphate-lyase (cyclique), EC 4.6.1.2) ont jusqu'à maintenant été identifiées.

3.1. Guanylyl cyclase soluble

La guanylyl cyclase soluble (GC-S) (Yuen et al., 1992) est un hétérodimère (sous-unités α M_r 60 kDa; sous-unité β M_r 70 kDa) (Kamisaki et al., 1986; Goy, 1991) à groupement hème (Gerzer et al., 1981) qui fût initiallement purifié à partir de poumon de rat (Garbers 1979). Le séquençage des sous-unités α et β de GC-S a révélé la conservation de 231 résidus pour une homologie de structure globale de 32%. La même étude a permis l'identification de six domaines de haute homologie avec les deux domaines hydrophiles de l'adénylyl cyclase de boeuf (Koesling et al., 1988; 1990). Fait à souligner, l'activité catalytique requiert la coexpression des deux sous-unités (Harteneck et al., 1990). Le modèle actuel veut que cette isoforme soit activée, via son groupement hème, par l'oxyde nitrique ou EDRF (*endothelium-derived relaxing factor*) généré par des vasodilatateurs comme l'acétylcholine, l'histamine, la substance P, la sérotonine et la vasopressine (Furchgott et al., 1989).

3.2. Guanylyl cyclase membranaire

La seconde isoforme réfère à une guanylyl cyclase membranaire initiallement isolée à partir de spermatazoïde d'oursin de mer (*Strongylocentrotus purpuratus*) (Garbers, 1976; Radany et al., 1983). Cette isoforme consiste en une protéine transmembranaire glycosylée cumulant les rôles de récepteur et d'enzyme, avec un domaine de ligation extracellulaire ainsi qu'un domaine cytoplasmique incorporant deux sous-domaines, soit un domaine d'homologie avec les protéines à activité tyrosine-kinases, et un site de catalyse de cGMP (Chinkers et Garbers, 1991; Yuen et al., 1992). Plusieurs sous-types rassemblés sous l'étiquette de la famille des récepteurs membranaires à activité guanylyl cyclase ont jusqu'à maintenant été identifiés et clonés. Outre le récepteur *S. purpuratus* (Mr 135 kDa) (Garbers, 1976; Radany et al., 1983), mentionnons l'existence du récepteur intestinal Sta (Mr 120 kDa) de l'entérotoxine de *Escherichia Coli* (GC-C) (Schultz et al., 1990), ainsi que les récepteurs natriurétiques (Mr 130 kDa) de type-A (GC-A ou NPR-A) (Lowe et al., 1989; Chinkers et al., 1989), et de type-B (GC-B ou NPR-B) (Chang et al., 1989; Schultz et al., 1989). NPR-A et -B sont responsables de la médiation de la majorité des effets physiologiques induits par les facteurs hormonaux de la famille des peptides natriurétiques. L'ANP et le BNP activent spécifiquement le NPR-A, alors que le CNP active spécifiquement le NPR-B (Koller et al., 1991)

4. Topographie des récepteurs natriurétiques

Il importe à ce stade-ci d'indiquer qu'outre NPR-A et -B, qualifiés de récepteurs biologiques R1 (Leitman et al., 1986), la famille des NPR comprend un troisième membre. Le récepteur natriurétique de type-C (NPR-C) (Fuller et al., 1988), classifié R2 par Leitman et al. (1986) est un récepteur homodimérique (Mr 130 kDa) composé de deux sous-unités (Mr 65 kDa) liées par un pont disulfure (Itakura et al., 1994) et vis-à-vis duquel tous les peptides natriurétiques affichent une haute affinité (Bennett et al., 1991). NPR-C se caractérise par un large domaine extracellulaire en amino-terminal, ancré par un passage transmembranaire simple terminé par un court segment cytoplasmique d'une quarantaine de résidus. NPR-C est dépourvue de toute activité catalytique intrinsèque et serait impliqué dans la destruction et/ou la réabsorption des peptides natriurétiques plasmatiques (Maack et al., 1987).

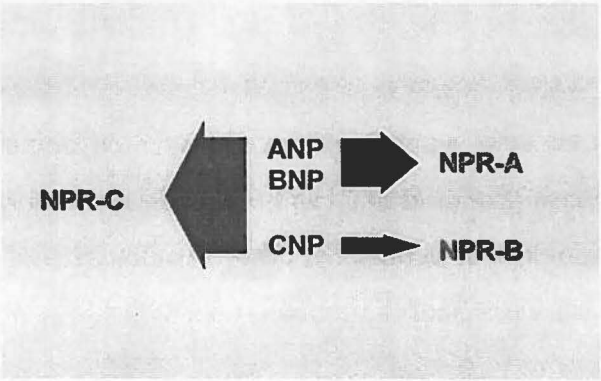


Figure 4. Les peptides natriurétiques interagissent avec trois récepteurs. ANP et BNP sont spécifiques à NPR-A, tandis que le CNP est spécifique au récepteur NPR-B. NPR-A et -B sont responsables de la médiation des effets diurétiques. Quant au récepteur NPR-C (vis-à-vis duquel les trois facteurs présentent une haute affinité) il est considéré comme récepteur de clairance. Ce récepteur serait également impliqué dans l'internalisation des facteurs natriurétiques.

4.1. Domaine extracellulaire

Le domaine extracellulaire (de 460 à 470 acides aminés) que partagent en amino-terminal les récepteurs NPR-A, -B et -C de rat, enregistrent des homologies de séquence de 43% entre -A et –

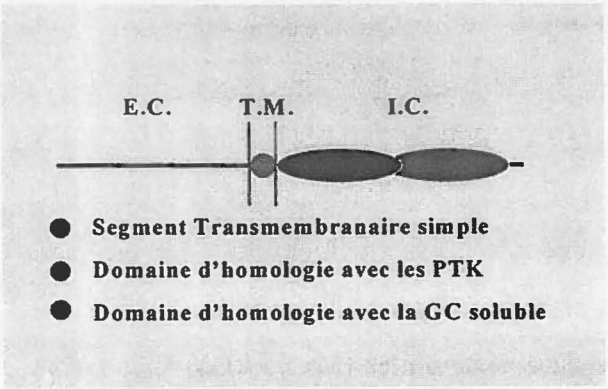


Figure 5. Schématisation de NPR-A et –B. (PTK, Protéine tyrosine kinase; GC, Guanylate cyclase).

B, 29% entre -B et -C (Schultz et al., 1989) et de 33% entre -A et -C (Chinkers et al., 1989; Lowe et al., 1989). NPR-A, -B, et -C, présentent respectivement 6, 6, et 5 cystéines dont cinq sont hautement conservées à travers tout le règne animal (Iwata et al., 1991). Récemment, McNicoll et al. on démontré que le fragment Asp191-Arg198 de NPR-A bovin correspondait à un site de liaison d'ANP (McNicoll et al., 1996).

4.2 Domaine cytoplasmique

NPR-C possède un court segment cytoplasmique dépourvu d'activité guanylyl cyclase. NPR-A et -B partagent un domaine cytoplasmique divisé en deux sous-domaines, à savoir un domaine proximal d'homologie aux protéines tyrosine kinase (Singh et al., 1988; Chinkers et al., 1989; Schultz et al., 1989), et un domaine distal d'homologie avec la guanylyl cyclase soluble (Koesling et al., 1988; Nakane et al., 1988), et l'adénylyl cyclase de boeuf (Krupinski et al., 1989).

4.2.1 Domaine d'homologie tyrosine-kinase

Chez NPR-A et -B de rat, ce domaine s'étend des résidus 518 à 776 et affiche une homologie de 72% (Schultz et al., 1989). Des 33 résidus invariables ou hautement conservés, propres aux protéines tyrosine-kinase identifiées par Hanks (Hanks et al., 1988), 22 sont conservés bien que le domaine soit catalytiquement inactif (Yuen et al., 1992). La délétion par mutagénèse de ce domaine entraîne une synthèse constitutive élevée de cGMP non influencée par l'ANP (Chinkers

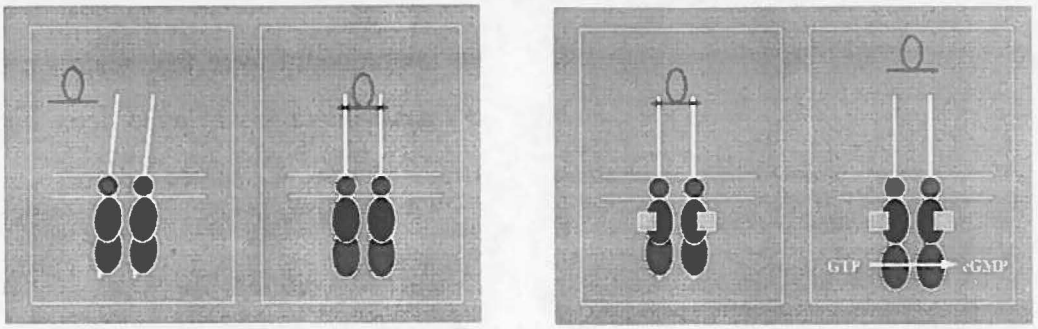
et al., 1989b). Un site de liaison d'ATP y est présent (Chinkers et al., 1989b; Larose et al., 1991) et la liaison du nucléotide entraîne une diminution de l'affinité d'ANP couplée à une augmentation de l'activité catalytique du récepteur. Ce domaine agirait en tant que régulateur négatif du domaine catalytique (Chinkers et al., 1989b).

4.2.2 Domaine d'homologie guanylyl cyclase

Chez NPR-A et -B de rat, ce domaine s'étend des résidus 804-1047 et partage une homologie de 91% (Schultz et al., 1989). Fait important à souligner, ce domaine catalytique partage une homologie de 40% avec le site catalytique de l'une des deux sous-unités de la guanylyl cyclase soluble (Thorpe et Garbers, 1989), et une homologie de 30% avec l'adénylyl cyclase (Koesling et al., 1988), deux cyclases dont l'activité catalytique repose sur une structure dimérique.

5. Dynamique de liaison: Modèle et problématique

La nature oligomérique des récepteurs natriurétiques R1, qui ont jusqu'ici été identifiés sous forme monomérique, dimérique et tétramérique, demeure sujet à controverse et l'objet de résultats contradictoires. Pareille problématique demeure une caractéristique propre à l'étude des protéines membranaires ou transmembranaires et aux difficultés techniques inhérentes à ce champ d'étude. Le cas des récepteurs natriurétiques R1 ne fait pas exception. Bien que des analyses par SDS-PAGE en condition non-réductrice aient effectivement sugéré la présence du récepteur NPR-A sous forme de dimère covalent (Shimonaka et al., 1986), pareil résultat est considéré comme un artefact puisque de nombreuses études similaires n'ont relevé que la présence du monomère (Yip et al., 1985; McNicoll et al., 1992; Leitman et al., 1987). D'ailleurs, la nature non-covalente des interactions existant entre sous-unités rend bien compte de l'instabilité des formes de haut poids moléculaire lorsque solubilisées dans des tampons à base de détergent. En effet, des analyses hydrodynamiques par centrifugation sur gradient de sucrose du récepteur NPR-A solubilisé dans le détergent non-ionique octyl-β-D-glucoside, n'ont permis de relever la présence que de la forme monomérique (110-130 kDa) (Meloche et al., 1986), alors que le méthode du FPLGC (*Fast Protein Liquid Gel Chromatography*), plus rapide, a permis de relever la présence de la forme tétramérique (Chinkers et al., 1992). Enfin, des analyses par tamis moléculaire sur gel ont signalé la présence de la forme monomérique et d'une forme de plus haut poids moléculaire (Meloche et al., 1987; Iwata et al., 1991). La difficulté de déterminer avec certitude les formes en présence, ainsi que les processus sous-jacents à leur modulation, rend difficile le développement d'un modèle satisfaisant. Néanmoins, et malgré le fait que demeure inconnu le mécanisme exact par lequel les récepteurs natriurétiques NPR-A et -B procèdent à la transduction du message, un concensus se dégage voulant que l'homodimérisation (ou encore l'hétérodimérisation NPR-A\NPR-B soit la forme de haute affinité et également la forme catalytiquement active (Chinkers et Wilson, 1992; Lowe, 1992; Rondeau et al., 1995). La figure 6 résume les grandes lignes du modèle actuellement retenu.



a **b** **c** **d**

Figure 6. Modèle transductionnel hypothétique. Par sa liaison au site de reconnaissance, le facteur hormonal stabilise l'homodimère en l'amenant de l'état **a** à **b**; dès lors, un site d'attache pour l'ATP devient accessible dans le domaine d'homologie tyrosine-kinase en **c**. Ceci libère le site catalytique de l'inhibition produite par le site d'homologie tyrosine-kinase, tout en diminuant l'affinité du ligand pour le récepteur. Il s'ensuit la production du second messager (cGMP)en **d**, puis le retour en **a**, par dissociation de l'ATP.

Le modèle qui se dégage résiste à l'analyse toporaphique des récepteurs natrurétiques R1. En effet, le fait que l'activité catalytique, tant de l'adénylyl cyclase que de la guanylyl cyclase soluble, requiert leur dimérisation préalable, combiné à l'observation d'un domaine d'homologie avec la guanylyl cyclase soluble chez NPR-A et -B, suggèrent fortement l'homodimérisation du site catalytique des récepteurs natriurétiques. Tout aussi significatif, la présence chez NPR-A et -B d'un seul segment transmembranaire, combiné à la présence d'un site d'homologie aux protéines tyrosine-kinases. Ceci suggère également leur dimérisation, en regard de l'homologie de structure avec les récepteurs de la famille des protéines à activité tyrosine-kinase, dont l'activité est dépendante d'une dimérisation, comme PDGFR (*platelet-derived growth factor receptor*), ou encore EGFR (*epidermal growth factor* receptor) (Heldin, 1995).

Si ces homologies aident à la validation du modèle, elles mettent également en lumière la possibilité que l'interaction ligand-récepteur, entre peptides natriurétiques et récepteurs R1, ne soit plus complexe qu'initialement anticipée, comme le propose les figures 7B et 7C. Fait

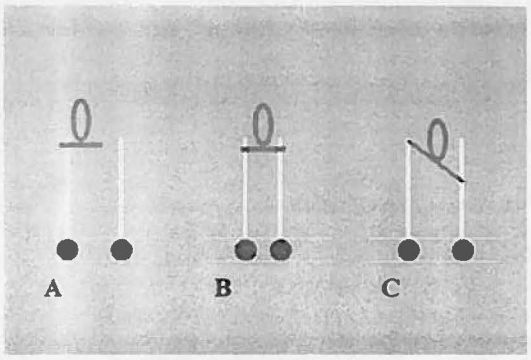


Figure 7. Trois interactions possibles, avec en **A** le ligand interagissant avec une seule sous-unité; en **B** le ligand interagissant avec deux domaines identiques, et en **C**, avec deux domaines différents.

intéressant à souligner, et qui concerne l'hormone de croissance, non seulement l'interaction GH/GHbp (*growth hormone/growth hormone binding protein*) respecte-elle une stoïchiométrie 2:1, mais aussi des analyses structurales par cristallographie ont permis de découvrir que GH interagit avec les deux sous-unités du récepteur (De Vos et al., 1992).

6. Prémisses et contexte de la recherche réalisée

C'est au début des années 90 que notre laboratoire et celui du docteur André De Léan ont initié un programme de recherche conjoint dont l'objet concerne la spécificité des peptides natriurétiques vis-à-vis leurs récepteurs respectifs. En regard des hautes homologies structurales existant entre les peptides natriurétiques au niveau de leur cycle, il a été alors décidé de procéder à la synthèse de chimères peptidiques (Fig. 8) et d'en évaluer le comportement pharmacologique, afin de juger de l'importance des segments exocycliques comme éléments restrictifs. Les travaux résultant (Mimeault et al., 1993) ont permis d'identifier un super-agoniste, le pBNP1 (pK_i = 11.26), dont l'affinité surpasse celles de l'ANP (pK_i = 10.71), et du BNP (pK_i = 10.44). Parallèlement à ce projet, et dans le but de cerner le site de reconnaissance du récepteur NPR-A bovin, le laboratoire du docteur De Léan amorce l'exploration de l'utilisation d'un dérivé benzophénone photoactivable incorporé dans une sonde issue de l'ANP pour le photomarquage du récepteur (McNicoll et al., 1992). Les résultats sont probants. L'utilisation de ce dérivé, le ^{125}I-[Tyr^2]rANP (2-27), permet non seulement le photomarquage du récepteur NPR-A bovin, mais également, par purification subséquente et séquençage, l'identification d'un site de reconnaissance. Tout est alors en place pour initier un nouveau projet de recherche.

rANP	**SLRRSSCFGGRIDRIGAQSGLGCNSFRY**
pBNP	**SPKTMRDSGCFGRRLDRIGSLSGLGCKVLRRH**
pBNP1	**SLRRSSCFGRRLDRIGSLSGLGCNSFRY**

Figure 8. Séquence primaire de l'ANP de rat (r), du BNP de porc (p), et de l'analogue chimérique [N, C, rANP(1-28)]pBNP32 (pBNP1). Comme le montre la figure, le cycle du BNP a été retenu alors que les segments exocycliques sont ceux de l'ANP. Les cystéines (C) sont impliquées dans un pont disulfure intramoléculaire.

7. Stratégie

Dans le but de mieux cerner la dynamique de liaison existant entre ANP\BNP et NPR-A, il a été décidé d'entreprendre un photomarquage d'affinité restreint, de façon à déterminer la nature des interactions existant entre le site de reconnaissance du récepteur et les segments cycliques et exocycliques du ligand (Article #2; Fig. 1).

L'étude nécessitant l'emploi de sondes de haute affinité, l'analogue chimérique pBNP1 devient vite le choix privilégié. De la même façon, la haute spécificité du benzophénone, ainsi que le taux important d'incorporation (70%) obtenu avec la sonde [Tyr^{116}, *p*-benzoyl-Phe^{125}]ANF(99-125) (McNicoll et al., 1992), font de cet agent photoactivable, là encore, le choix privilégié.

Ainsi, pour la première fois:

le pBNP1 a été utilisé comme gabarit de synthèse pour le développement de sondes de haute affinité, en conjonction avec le benzophénone comme agent de réticulation de haute spécificité.

un «benzophénone-scan» englobant tous les domaines de l'hormone a été réalisé de façon à permettre une analyse détaillée des points de contact ligand/récepteur.

Et enfin, des profils de digestion au bromure de cyanogène du récepteur complexé au ligand ont été réalisés de façon à comparer les profils obtenus, et à fournir des indices quant aux segments protéiques du récepteur impliqués dans la liaison.

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Seconde Partie

1.1. Résumé français de l'article #1

Titre

Synthèse de sondes *p*-benzoylbenzoylées dérivées de l'analogue [N,C,rANF(99-126)]pBNP32 (pBNP1) pour le photomarquage d'affinité du récepteur NPR-A bovin.

Résumé

Plusieurs résultats suggèrent l'existence d'une interaction entre le segment exocyclique situé en amino-terminal de l'ANP (*atrial natriuretic peptide*) et l'une des sous-unités délimitant le site de reconnaissance du récepteur homodimérique NPR-A (*natriuretic peptide receptor type-A*). Dans le but de pousser plus avant l'étude de cette possible interaction, nous avons procédé à la synthèse sur support solide de deux sondes photoactivables de haute spécificité, dérivées de l'analogue chimérique de haute affinité [N,C,rANP(1-28)]pBNP32 (identifié comme le pBNP1) (Mimeault et al., 1993, *Mol. Pharmacol. 43*, 775-782), et incorporant un benzophénone en amino-terminal. [N^{α}-*p*-benzoylbenzoyl, Tyr^{100}]pBNP1, et [N^{α}-*p*-benzoylbenzoyl, Tyr^{116}]pBNP1 ont été synthétisés par chimie Boc/BOP. Le BOP a également été utilisé comme agent de couplage pour la dérivation en N^{α}. Les sondes ont été caractérisées par HPLC analytique, électrophorèse capillaire et spetrométrie de masse. Les potentiels de liaison ont été évalués sur des préparations membranaires de cortex de glande surrénale de boeuf. Les deux sondes ont affiché des affinités statistiquement identiques à celle démontrée par l'ANF (K_d = 20 pM) avec des K_d de 10 pM et de 30 pM pour [N^{α}-*p*-benzoylbenzoyl, Tyr^{100}]pBNP1, et [N^{α}-*p*-benzoylbenzoyl, Tyr^{116}]pBNP1, respectivement. L'utilisation de la lactoperoxydase pour l'iodation des sondes s'est soldée par une iodation préférentielle du résidu Tyr^{126}, celui-ci ayant incorporé 80%-90% du signal radioactif total. Comparativement à seulement 3% pour ^{125}I-pBNP1, la liaison non-specifique s'est

montrée élevée, correspondant respectivement à 34% et 22% pour [N^{α}-*p*-benzoylbenzoyl, Tyr^{100}]pBNP1, et [N^{α}-*p*-benzoylbenzoyl, Tyr^{116}]pBNP1. Les deux sondes se sont montrées efficaces pour le photomarquage spécifique d'une protéine de 130 kDa correspondant à la forme monomérique du récepteur NPR-A. Les taux d'incorporation irréversibles ont été estimés respectivement à 18% et 41% pour [N^{α}-*p*-benzoylbenzoyl, Tyr^{100}]pBNP1, et [N^{α}-*p*-benzoylbenzoyl, Tyr^{116}]pBNP1. L'addition de l'agent antihypertensif guanabenz (1 mM) au tampon de liaison a permis d'augmenter les taux d'incorporation à 29% et 52%, respectivement.

Synthesis of *p*-benzoylbenzoylated [N,C,rANP(1-28)]pBNP32 (pBNP1) derivatives and affinity photolabeling of the bovine NPR-A receptor.

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List of abbreviations

Acm, acetamidomethyl; ANP, atrial natriuretic peptide; Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; BrZ, 2-bromobenzyloxycarbonyl; DIPEA, diisopropylethylamine; DMF, N,N-dimethylformamide; NPR-A, natriuretic peptide receptor type A; NPR-B, natriuretic peptide receptor type B; OBzl, benzyl ester; pBNP32, porcine brain natriuretic peptide; pBNP1, [N,C,rANP(1-28)]pBNP32; Tos, *p*-toluenesulfonyl.

Abstract

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. Previous results obtained by our laboratory (Rondeau *et al.*, Biochemistry, 34, 2130, **1995**) suggested that this could be also the case with ANP, where the exocyclic amino and carboxyl segments might each interact with one of the subunits delineating the binding domain of the dimeric NPR-A. In order to further investigate this purported amino-terminal/NPR-A interaction, two highly specific N^{α}-benzophenone-substituted photoprobes, derived from the high affinity NPR-A chimeric agonist pBNP1 (Mimeault *et al.*, Mol. Pharmacol., 43, 775, **1993**) were assembled by solid-phase peptide synthesis. [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 were synthesized using the Boc/BOP strategy. BOP was also used as the coupling reagent of *p*-benzoylbenzoic acid for both N^{α}-derivatization. Purity of the peptides was assessed by analytical HPLC, capillary electrophoresis and mass spectrometry. The affinity of the analogs was tested on bovine *zona glomerulosa* membrane preparations and found to exert ANP-type high affinities (K_d = 20 pM) with K_d of 10 pM and 30 pM for [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively. Peptide iodination using the lactoperoxydase procedure was found to be highly preferential to Tyr^{28} as it incorporated 80%-90% of the total radioactive signal. As compared to only 3% for ^{125}I-pBNP1, non-specific binding was elevated, corresponding to 34% and 22% for [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively. Photolabeling of NPR-A with both analogs cross-linked specifically a 130 kDa monomeric protein. The maximal irreversible ligand incorporations were estimated at 18% and 41% for [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively. The addition of guanabenz (1 mM) to the binding buffer increased the irreversible incorporations to 29% and 52%, respectively.

Keywords: Benzophenone, solid-phase peptide synthesis, photoaffinity labeling, ANP, BNP, pBNP1, NPR-A.

Introduction

Natriuretic peptides mediate most of their homeostatic cardiovascular effects (Lewicki and Protter, 1995; McDowell et al., 1995 *for reviews*) through cellular accumulation of cyclic guanosine 3' 5' monophosphate (Leitman and Murad, 1987). Signal transduction is brought upon ligand-binding dependent activation of the particulate guanylate cyclase R1 receptors (Anand-Srivastava and Trachte, 1993; Yuen and Garbers, 1992 *for reviews*). Two R1 cDNA subtypes have so far been cloned from various species. They are designated as NPR-A (Chinkers et al., 1989; Lowe et al., 1989) which is activated by ANP and to a 10-fold lesser extent by BNP (Suga et al., 1992; Schulz et al., 1989), and NPR-B (Chang et al., 1989; Schulz et al., 1989) which is specific to CNP (Koller et al., 1991). Both receptors possess an apparent M_r of 130 kDa (Meloche et al., 1988; Takayanagi et al., 1987; Kuno et al., 1986) and share the same overall topology characterized by a large extracellular binding domain, a single transmembrane domain, and a large cytoplasmic domain divided in two critical subdomains: a 63% identical proximal membrane segment displaying 30% homology with the protein kinase domain of the growth factor receptor family, and a 88% identical distal domain displaying a 40% homology with soluble guanylyl cyclase (Koller and Goeddel, 1992).

In accordance with the dimeric nature of both the growth hormone receptor and soluble guanylyl cyclase, NPR-A was shown by steric exclusion chromatography (Meloche et al., 1987) and by chemical cross-linking experiments (Iwata et al., 1991) 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 (Rondeau et al., 1995). Recently the high efficiency (>70%) labeling probe [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (McNicoll et al., 1992), which permitted identification and localization of a portion of the NPR-A binding domain (McNicoll et al., 1996), was derived into the bifunctional probe [*p*-azidobenzoyl-Ser^{1}, Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (Rondeau et al., 1995). Photoaffinity labeling of bovine adrenal *zona glomerulosa* NPR-A with this bifunctional probe provided evidence that the amino- and carboxyl-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. These results are corroborated by structure-activity relationship studies showing that removal in ANP of Arg^3 and Arg^4 leads to a 10-fold reduction in affinity toward bovine adrenal *zona glomerulosa* NPR-A (De Léan et al., 1985) and a 4-fold reduction in cultured bovine aortic smooth muscle cells (Scarborough et al., 1986). These results suggested a minor but non-negligible interacting role for the N-terminal segment with the receptor binding domain. Noteworthy, satisfying yields were obtained for the NPR-A purification by ANP-affinity chromatography when using a matrix with the hormone coupled by its more critical C-terminal end (Meloche et al., 1988). 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 amino- 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%) (Pilch and Czech, 1983) were circumvented by replacing it with the highly site specific and efficient benzophenone photophore (Dorman and Prestwich, 1984). Furthermore, the high affinity 28-residue hybrid natriuretic analog [N,C,rANP(1-28)]pBNP32 (K_d = 8pM) (Mimeault et al., 1993) obtained by combining the ring structure of pBNP32 (K_d = 36pM) with the amino- and carboxyl segment of rANP(1-28) (K_d = 20pM) was used as the template in order to increase 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} (Carpenter et al., 1997; Fairbrother et al., 1994). Single substitution experiments of ANP(3-28) produced low potency analogs when Phe^8, Ile^{15}, or Leu^{21} were replaced by alanine residues (Bovy, 1990). Similar experiments done on hANP have determined the prime importance of

Phe[8], Asp[13], Arg[14], Ile[15], Leu[21], and Arg[27] (Li et al., 1995). The same residues were found important when an alanine scan was performed on rANP(3-28) (Bovy, 1990), 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 based on surface accessibility shown by NMR molecular modeling of pBNP1 (Carpenter et al., 1997) and also on the NMR modeling of the ANP hexamutant ([R3D, G9T, R11S, M12L, R14S, G16R]hANP(1-28)) (Fairbrother et al., 1994). The hydrophobic nature of both residues was also considered favorable.

Thus we report the synthesis of the high affinity labeling probes [N^{α}-*p*-benzoylbenzoyl, Tyr[2]]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr[18]]pBNP1. These derivatives display higher incorporation efficiency as compared to cross-linking or affinity cross-linking equivalents.

Experimental procedures

Materials for peptide synthesis. Boc-amino acids were from Chem-Impex International (Wood Dale, IL) and *p*-benzoylbenzoic acid was from Sigma Canada Ltd. (Mississauga, Ontario, Canada). BOP was from Richelieu Biotechnologies (Montréal, Québec, Canada). ACS-grade dimethylformamide and methylene chloride were from Anachemia Canada inc. (Ville St-Pierre, Québec, Canada). Diisopropylethylamine and cesium hydrogen carbonate were from Aldrich (Mississauga, Ontario, Canada). Chloromethylated resin (copolystyrene-1% divinylbenzene, 0.75 meq/g) was from Bio-Rad Laboratories Canada Ltd. (Mississauga, Ontario, Canada). Biograde trifluoroacetic acid was from Halocarbon (Hackensack, NJ).

Materials for peptide characterization and photoaffinity labeling. Bovine kidneys were obtained from a local slaughterhouse. Aprotinin, leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF) were from Sigma Canada Ltd. (Mississauga, Ontario, Canada). Carrier-free $Na^{125}I$ was from Amersham Life Science Inc. (Arlington Heights IL). Bovine serum albumin, carboxypeptidase A, sodium dodecyl sulfate, tris(hydroxymethyl)aminomethane and glycine were from Boehringer Mannheim Canada (Laval, Québec, Canada). Electrophoresis reagents and standards were from Bio-Rad Laboratories Canada Ltd. (Mississauga, Ontario, Canada). GF/C filters were obtained from Fisher Scientific Canada (Montréal, Québec, Canada). Poly(ethyleneimine) was from Aldrich (Mississauga, Ontario, Canada).

Boc-Tyr(BrZ) Resin. Boc-Tyr(BrZ) (34 mmoles) was dissolved in ethanol (100 ml) and water (40 ml) and treated with 40 ml of a 2M aqueous solution of hydrogen cesium bicarbonate to give a final pH of 7.0. The clear solution was evaporated under vacuum and then coevaporated with toluene (3 X 100 ml) to give a white solid. This residue was dried under vacuum for 24 h. The chloromethylated resin (15 g, polystyrene-1% divinylbenzene; substitution 0.75 mmol/g), the cesium salt of Boc-Tyr(BrZ), and KI (1.5g) were stirred in dimethylformamide (5 ml) with a mechanical stirrer at 50°C for 24 h. The resin was then washed with dimethylformamide (3 x 100 ml), dimethylformamide-H_2O (1:1, 3 x 100 ml), methanol (3 x 100 ml) and methylene

chloride (3 x 100 ml). The resin was then dried under high vacuum to give a final weight of 21.3 g. The substitution measured with the picric acid procedure (Gisin, 1972) was evaluated at 0.5 mmol/g.

Peptide synthesis. All analogs were synthesized by the solid-phase peptide synthesis strategy (Merrifield, 1964) using a homemade manual multireactor system and following a Boc chemistry protocol. A quantity of 2.5 g of Boc-Tyr(BrZ) resin (substitution 0.5 mmol/g) was used per reactor. Couplings were carried out with 3 eq. of BOP reagent, 3 eq. of Boc-AA-OH and 5 eq. of diisopropylethylamine. A complete coupling cycle is given in Table 1. Reaction monitoring was achieved using the ninhydrin test (Kaiser et al., 1970). Amino acid side chain deprotection, as well as the peptide cleavage from the solid support were achieved 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 followed by evaporation.

Peptide purification. All crude analogs were purified by preparative reverse-phase HPLC on a Waters Prep LC 500A system equipped with a Waters 1000 Prep Pak Module and a 441 absorbance detector. Peptide solutions (700 mg in 0.06% TFA/H_2O) were injected on a DeltaPak C_{18} (15 µm, 30 nm) reverse-phase column (30 cm x 4.7 cm). The material was eluted with a linear gradient of (A) 0.06% TFA/H_2O and (B) acetonitrile (35%) in 0.06% TFA/H_2O, as follows: 0% of B to 100% B in 90 min. Flow rate was set at 60 ml/min and detection was performed at 230 nm. Individual 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} (10 µm) reverse-phase column (30 cm x 0.39 cm) and an eluate of (A) 0.06% TFA/H_2O and (B) acetonitrile in a linear gradient mode (15% B to 45% B in 15 min). Flow rate was set at 1.5 ml/min and detection was performed at 230 nm. Additional analyses were made using a Pharmacia Super Pak C_{18} (5µm) reverse-phase column (25 cm x 0.4 cm) and an eluate of (A) 0.06% TFA/H_2O and (B) acetonitrile in a

linear gradient mode (20% B to 40% B in 15 min) at a flow rate of 1 ml/min. Detection was performed at 230 nm.

Peptide cyclization. Formation of the disulfide bridge was carried out in 10 ml of a degassed 80% acetic acid solution containing 50 mg of the lyophilized purified linear peptide and 200 mg (50 eq) of I_2. The reaction was carried out for 30 min before being neutralized with a minimal amount of ascorbic acid until complete solution discoloration. The resulting cyclic peptide was then purified using the procedure described above.

Preparation of membranes. Bovine adrenal *zona glomerulosa* membranes were prepared as previously described (Meloche et al., 1986). 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 (Rockford, IL).

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. 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 $Na^{125}I$ 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 □m nylon 66 Conz Syringe-Tip filter, and the iodinated peptide purified on a Vydac C_{18} (10µm) 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. 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 (McNicoll et al., 1991). Radioligand binding was achieved as stated above. At the end of the incubation period the scavenger *p*-aminobenzoic acid was added (10^{-7} M) to the binding mixture. 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 (De Léan et al., 1982; De Léan et al., 1978).

Results

Peptide synthesis and purification. Human ANP(1-28) and the analogs [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 were synthesized in our laboratories (Figure 1). All peptides were synthesized using BOP as the coupling reagent (Castro et al, 1975) according to previously described synthetic methods (Fournier et al., 1988). The N-terminal derivatization was achieved in situ with BOP as the coupling reagent of *p*-benzoylbenzoic acid. Similar strategies have been reported using dicyclohexylcarbodiimide as the coupling reagent (Thiele and Fahrenholz, 1993; Parker and Hodges, 1985). Following hydrogen fluoride cleavage, both analogs gave very good peptide preparations. The reported difficulties for the assemblage of the hANP sequence Arg^{11}-Ile^{15} (Balasubramanian et al., 1984) were not encountered, even in the synthesis of hANP(1-28), stressing, as expected, the advantages of the BOP reagent method over the dicyclohexylcarbodiimide coupling approach. 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 10 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 fast atom bombardment mass spectrometry (m/z 3421.2 ± 0.5 and 3421.5 ± 0.6 for [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively; expected: 3420.9 in both cases).

Binding assays. Bovine adrenal *zona glomerulosa* membrane preparations were used to assess the potencies of both analogs. Choice was based on the fact that this tissue provides a simple, sensitive and reliable model which has been thoroughly investigated. As a matter of fact, bovine adrenal *zona glomerulosa* NPR-A has been characterized (De Léan et al., 1984), detergent solubilized (Meloche et al., 1986), purified to homogeneity (Meloche et al., 1988) and its glycoprotein nature was demonstrated (Liu et al., 1989). This tissue preparation was also found to be rich in the NPR-A receptor subtype (Meloche

et al., 1988). Affinities were determined by competitive binding assays against ^{125}I-rANP(1-28). As shown in figure 2, both analogs were able to compete against ^{125}I-rANP(1-28), displaying competition binding curves with slope factor close to unity (Table 2), as expected with a model involving a single class of receptor sites. As shown by the pK_i values in Table 2, both analogs in their linear form present very low potencies relative to rANP(1-28) ($\leq$ 1%), stressing the importance of the disulfide-link (Koide et al., 1993; Bovy, 1990). 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 [N^{α}-*p*-benzoylbenzoyl, Tyr^2]pBNP1 and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively, compared to 10.71 ± 0.10 for rANP(1-28) (Table 2).

Iodination of analogs. Purification of ^{125}I-[N^{α}-*p*-benzoylbenzoyl, Tyr^2]pBNP1 and ^{125}I-[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 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 [N^{α}-*p*-benzoylbenzoyl, Tyr^2]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, 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, it soars to 34% for [N^{α}-*p*-benzoylbenzoyl, Tyr^2]pBNP1, and to 22% for [N^{α}-*p*-benzoylbenzoyl,

Tyr[18]]pBNP1, as computed by analysis of the saturation curves (Figure 3). Interestingly, a high concentration (10^{-6} M) of the unlabeled analog [N^{α}-*p*-benzoylbenzoyl, Tyr[18]]pBNP1 was shown to cut non-specific binding of its labeled counterpart by more than half (data not shown). Also, addition in the binding medium of 1 mM of guanabenz increased total binding without affecting non-specific binding (Figure 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 (Figure 5). Maximum yield of incorporation for [N^{α}-*p*-benzoylbenzoyl, Tyr[2]]pBNP1 (≈1700 cpm/20 μg of protein) was set at 18% of the total specifically bound radioligand. Maximum yield of incorporation for [N^{α}-*p*-benzoylbenzoyl, Tyr[18]]pBNP1 (≈3300 cpm/20 μg of protein) was set at 41% of the total specifically bound radioligand.

Discussion

We have synthesized the photoaffinity probes [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 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 [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, 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 non-interfering nature of the benzophenone photophore as well as the tyrosine substitution is consistent with several structure-activity relationship studies. Thus, it has been demonstrated that the extension of the N-terminal of ANP, up to Glu^{54} (proANP(54-126)), reduced only slightly both receptor affinity and biological activity in *zona glomerulosa* cells (De Léan et al., 1985). Similarly, BNP and its N-terminal extended form BNP32 were shown to share the same relaxant potencies on chick rectum (Sudoh et al., 1988) suggesting again a lack of major structural constraints in the N-terminal region of both natriuretic peptides. A recent NMR study of pBNP1, carried out by our research group, concluded at the random coil nature of the N-terminal segment, even in presence of the membrane mimetic surface agent dodecylphosphocholine (Carpenter et al., 1997). NMR studies carried out with ANP in membrane-simulating agent reached the same conclusion (Koyama et al., 1990; Kobayashi et al., 1988).

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 [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, 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 (Mimeault et al., 1993; McNicoll et al., 1992), the addition of the N-terminal photophore causes a drastic effect on the hydrophobicity, especially in the case of [N^{α}-*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 (Trainer et al., 1997). Interestingly, non-specific binding of ^{125}I-[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 measured in presence of a high concentration (10^{-6} M) of unlabeled rANP(1-28) or pBNP1 did not alter results while at the same concentration unlabeled [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 reduced non-specific binding by half (data not shown). These results suggest high non-specific binding of the hydrophobic probe to the membrane vesicular surfaces, a well documented behavior of hydrophobic peptides (Sargent et al., 1989; Gysin and Schwyzer, 1984). Reported addition of 0.01% Triton X-100 in the binding medium in order to minimize non-specific binding (Trainer et al., 1997) did not modify results. We found that the problem can be offset using the antihypertensive agent guanabenz (Brochu et al., 1991). We observed that addition of 1 mM of this agent amplifies total binding without affecting non-specific binding, resulting in a overall increase of specific binding (Figure 4). Guanabenz is known to be almost equipotent to amiloride in increasing ANP affinity toward bovine adrenal zona glomerulosa NPR-A (De Léan, 1986). In fact, both species share a similar structure featuring a guanidinium moiety that is thought to interact with an allosteric binding site on the receptor protein (Meloche et al., 1988).

Computed incorporation efficiency percentages stand at 18% and 41% for [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, respectively. These yields are different to those of [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (70%)

(McNicoll et al., 1992) or other reported benzophenone-modified ligands such as [Bpa^{8}]Substance P (70%) (Boyd et al., 1991) or the myosine light-chain kinase analog [Bpa^{3}]MLCK (70%-90%) (Kauer et al., 1986). 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 (Dorman and Prestwich, 1994). Bearing in mind the somewhat random coil 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%) (Pandey et al., 1986; Misono et al., 1985; Yip et al., 1985), bifunctional reagents (5% - 10%) (Pilch and Czech, 1983), or underivatized ^{125}I-rANP(1-28) (1% - 7%) (Larose et al., 1990). As expected, presence of guanabenz (1 mM) in the binding buffer, by increasing specific binding also increased significantly the incorporation of ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and ^{125}I-[N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, to 29% and 52%, respectively (data not shown).

By conducting these studies we have underline the synthesis feasibility of both [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, and showed their high affinity. Both probes confirm previous results suggesting a participating role for ANP amino exocyclic segment in the ligand binding dynamic (Rondeau et al., 1995). 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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Figure legends

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.

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 (20μg) were 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^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (**A**), and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, (**B**). Curves (n=3) were analysed by the computer program ALLFIT for nonlinear lest-square regression based on a generalized form of the law of mass action (De Léan et al., 1978).

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) were 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 analysed 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 is reported for cyclic [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1 (**A**), and [N$^{\alpha}$-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1, (**B**).

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, then centrifuged, the pellet 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.

Figure 1.

Peptide	Structure
rANP(1-28)	SLRRSSCFGGRIDRIGAQSGLGCNSFRY
pBNP32	SPKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
pBNP1	SLRRSSCFGRRLDRIGSLSGLGCNSFRY
[N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1	*p*-benzoylbenzoyl-SYRRSSCFGRRLDRIGSLSGLGCNSFRY
[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1	*p*-benzoylbenzoyl-SLRRSSCFGRRLDRIGSYSGLGCNSFR

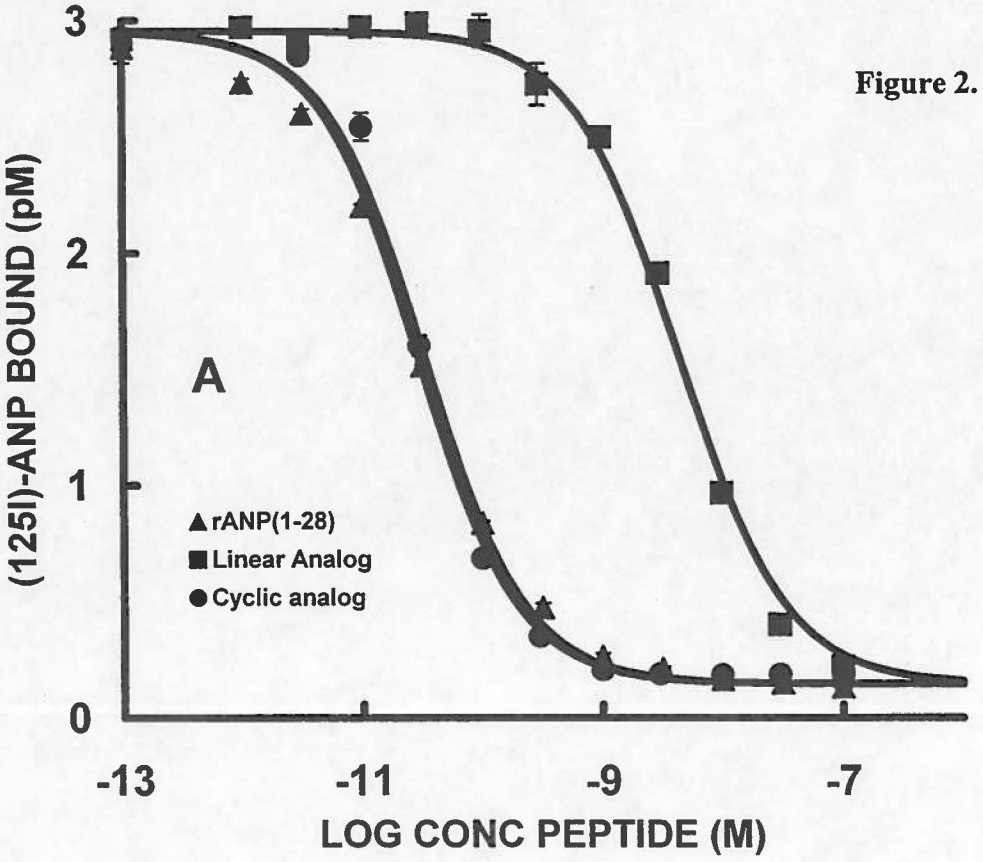
3
2
1
0
(125I)-ANP BOUND (pM)
A
▲rANP(1-28)
■Linear Analog
●Cyclic analog
-13
-11
-9
-7
LOG CONC PEPTIDE (M)

Figure 2.

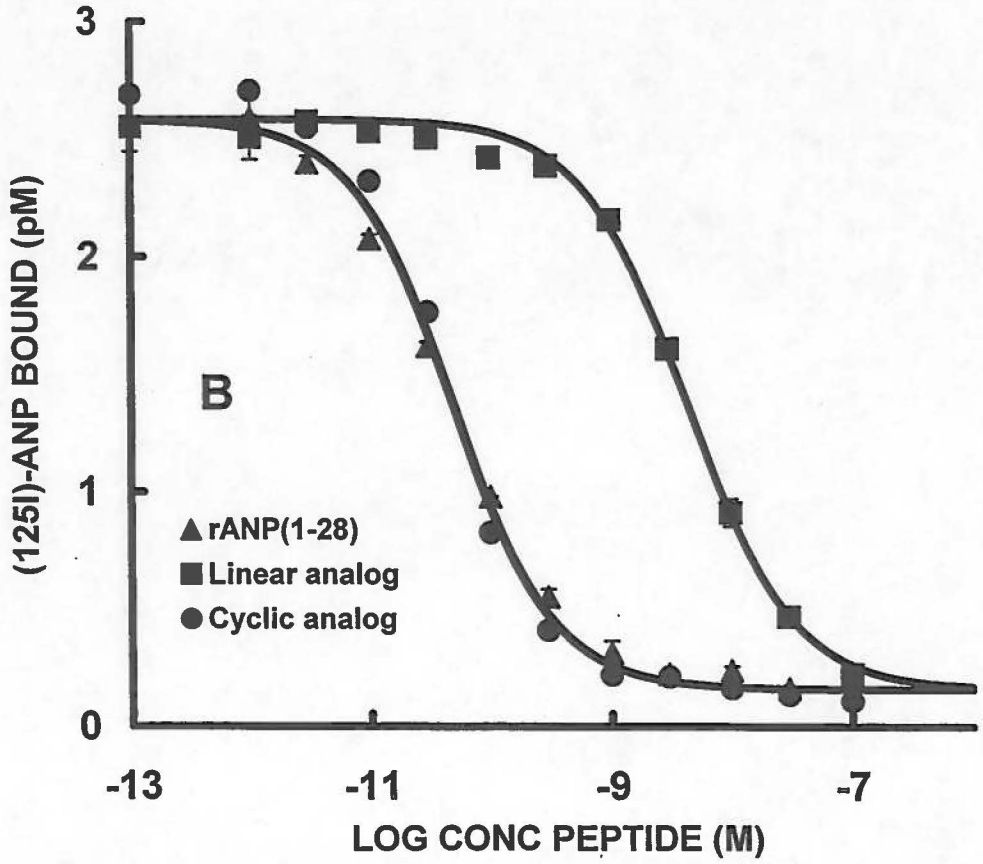
3
2
1
0
(125I)-ANP BOUND (pM)
B
▲rANP(1-28)
■Linear analog
●Cyclic analog
-13
-11
-9
-7
LOG CONC PEPTIDE (M)

Figure 3.

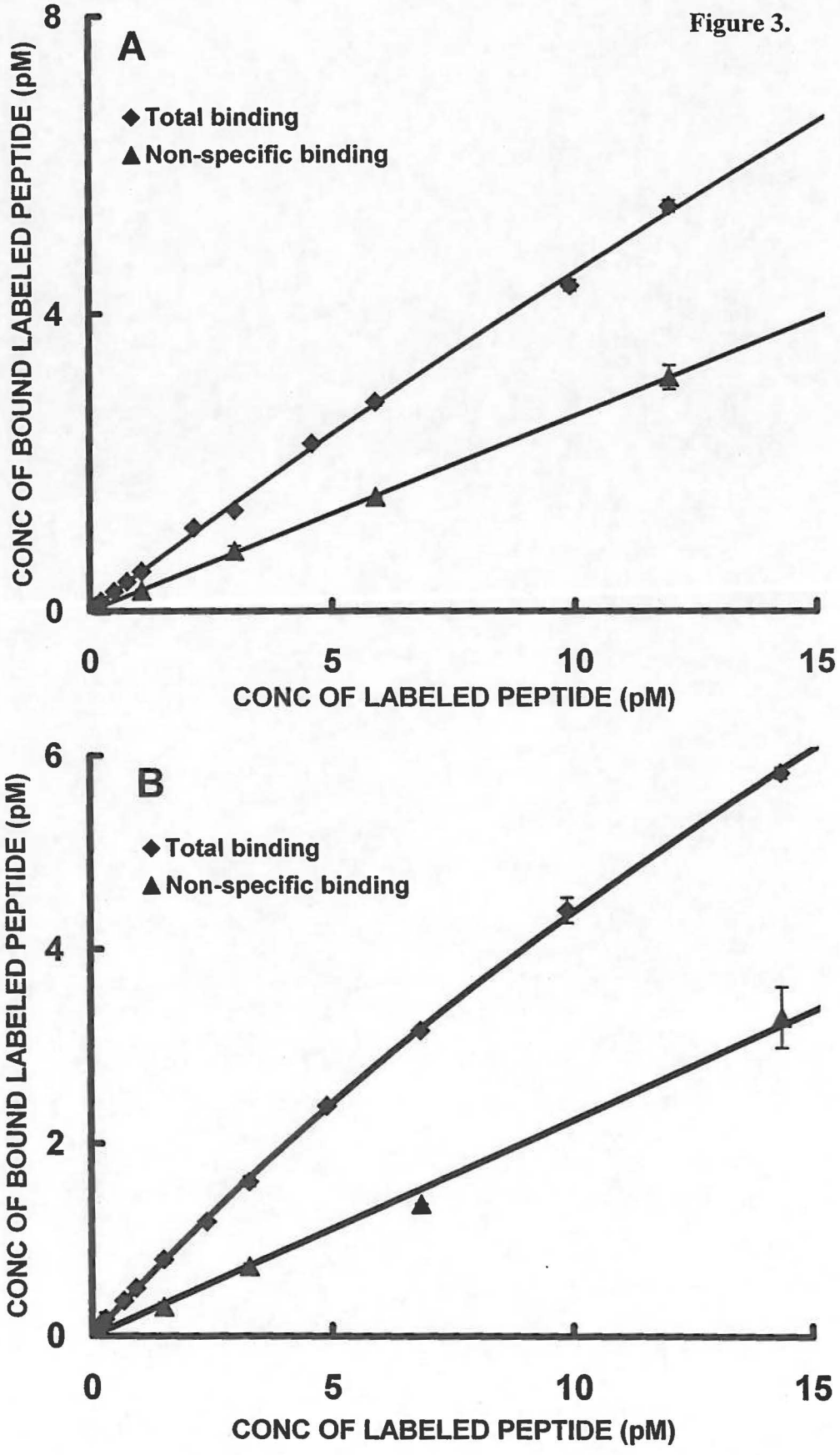
A
◆ Total binding
▲ Non-specific binding
CONC OF BOUND LABELED PEPTIDE (pM)
8
4
0
0
5
10
15
CONC OF LABELED PEPTIDE (pM)
B
◆ Total binding
▲ Non-specific binding
CONC OF BOUND LABELED PEPTIDE (pM)
6
4
2
0
0
5
10
15
CONC OF LABELED PEPTIDE (pM)

Figure 4

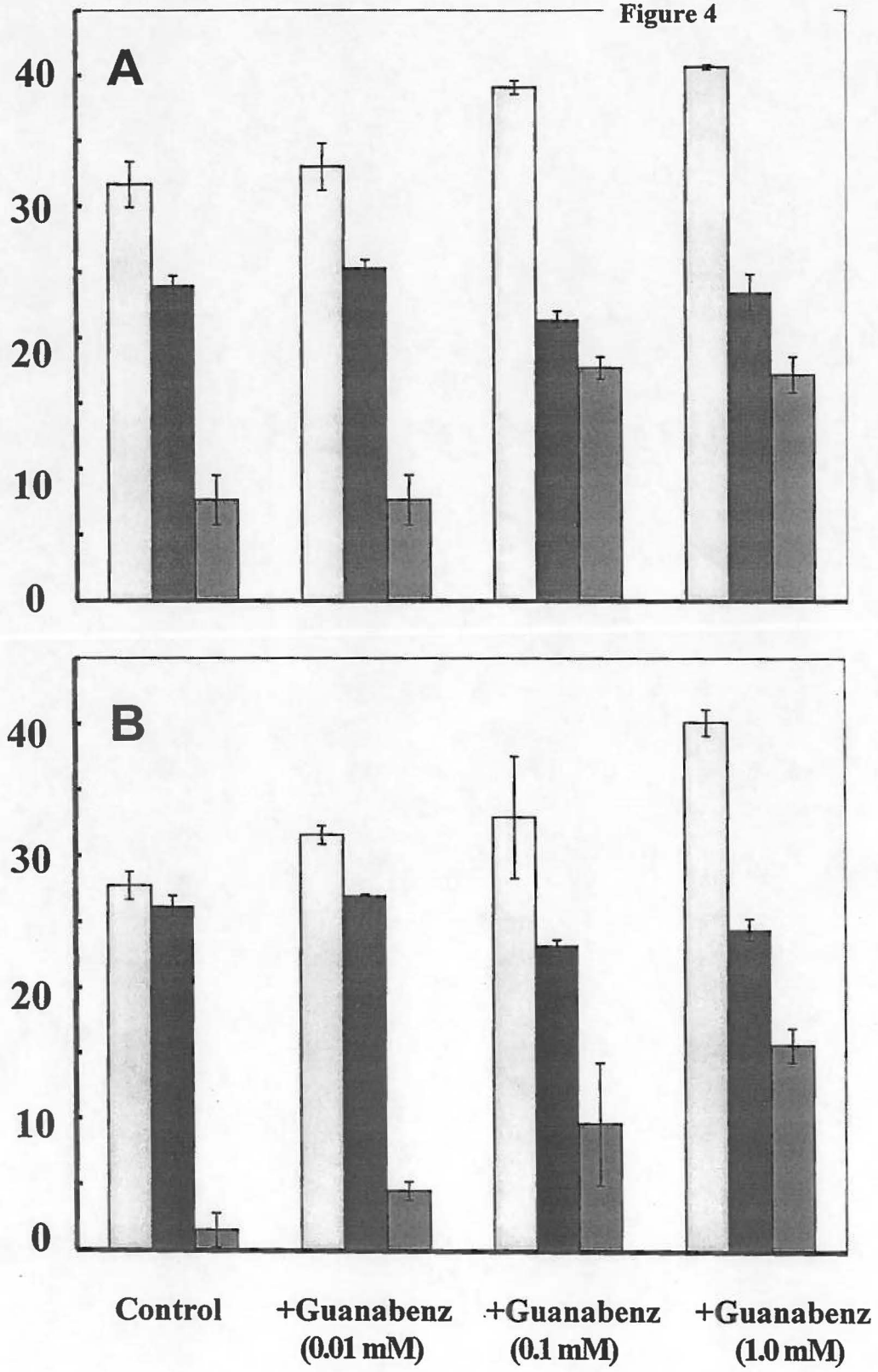
A
40
30
20
10
0
B
40
30
20
10
0
Control
+Guanabenz (0.01 mM)
+Guanabenz (0.1 mM)
+Guanabenz (1.0 mM)

Figure 5.

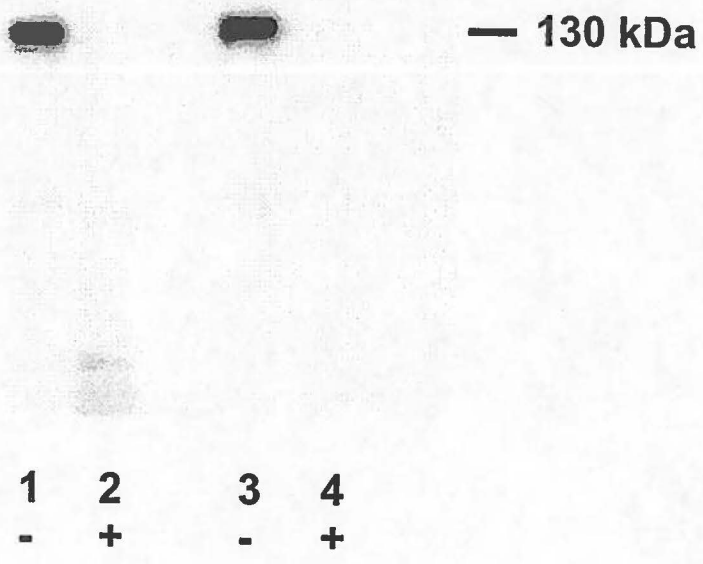

Table 2. NPR-A affinities of rANF(99-126), [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1, and [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1 in bovine adrenal *zona glomerulosa* membrane preparations. Affinities are given as pK_i (-log K_d); mean ± standard error of two to four receptor binding assays with ^{125}I-rANF(99-126).

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 ± 0.03	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

Table 1. Protocol for a synthetic cycle using BOP reagent. Solvent for all washings and couplings were measured to volumes of 10 - 20 ml/g of resin. Couplings were usually achieved in 45 min or less. In the case N^{α}-derivatization, 3 eq. of *p*-benzoylbenzoic acid were used.

Step	Reagent	Time
1	CH_2Cl_2	2 x2 min
2	40% CF_3CO_2H / CH_2Cl_2	1 x 5 min
3	40% CF_3CO_2H / CH_2Cl_2	1 x 20 min
4	CH_2Cl_2	2 x 2 min
5	EtOH	1 x 2 min
6	CH_2Cl_2	1 x 2 min
7	1% DIEA / DMF	1 x 2 min
8	DMF	1 x 2 min
9	3 eq. of Boc-AA-CO_2H / DMF 3 eq. of BOP reagent + 5 eq. of DIEA	45 min or less
10	DMF	2 x 2 min
11	CH_2Cl_2	1 x 2 min

2.1. Résumé français de l'article #2

Titre

Photomarquages régio-spécifiques du récepteur NPR-A bovin par l'incorporation de l'agent photoactivable benzophénone dans des sondes de haute affinité dérivées du [N,C,rANP(1-28)]pBNP32 (pBNP1)

Résumé

Dans le but de mieux cerner la dynamique des interactions existant entre les peptides natriurétiques et le récepteur natriurétique de type-A, nous avons procédé à des études systématiques de photomarquage de haute affinité avec le benzophénone comme agent photoactivable, et l'analogue chimérique de haute affinité [N,C,rANP(1-28)]pBNP32 (identifié comme le pBNP1) (Mimeault et al., 1993, *Mol. Pharmacol. 43*, 775-782), comme gabarit de synthèse.

Huit sondes ont été synthétisées sur support solide, soit: [N^{α}-*p*-benzoylbenzoyl, L2Y]pBNP1 (A); [N^{α}-*p*-benzoylbenzoyl, L18Y]pBNP1 (B); [L2Y, R3Bpa]pBNP1 (C); [S6Y, G9Bpa]pBNP1 (D); [G16Bpa, L18Y]pBNP1 (E); [L18Bpa, F26Y]pBNP1 (F); [L18Y, F26Bpa]pBNP1 (G); et [L18Y, Y28Bpa]pBNP1 (H). Les potentiels de liaison ont été évalués sur des préparations membranaires de cortex de glande surrénale de boeuf. Sous leur forme linéaire, tous les peptides ont démontré une faible affinité. À l'inverse, les analogues cycliques pBNP1-A, -B, -C, -F, -G, et -H ont enregistré des affinités statistiquement équivalentes à celle démontrée par l'ANP (Δ pK_i : de -0.04 à +0.14), alors que pBNP1-D (Δ pK_i = -0.43 ± 0.10) a enregistré une affinité 3 fois moindre. Considérant sa faible affinité, pBNP1-E (Δ pK_i = -1.76 ± 0.08) a été rejeté comme sonde potentielle. L'iodation des analogues via l'utilisation de la lactoperoxydase s'est caractérisée par l'iodation préférentielle du résidu Tyr^{28}. De l'incorporation du benzophénone au niveau du segment N-terminal (pBNP1-A, -B, -C) ont découlé les sondes affichant les plus importants taux

de liaison non-spécifique, situés à 34%, 22%, et 20%, respectivement, alors que des substitutions faites à l'intérieur du cycle (pBNP1-E, -F), et dans le segment exocyclique C-terminal (pBNP1-G, -H), ont decoulé des sondes affichant des taux de liaison non-spécifique situés à 15%, 14%, 7%, et 6%, respectivement. Les sondes ^{125}I-pBNP1-A, -B, -C, -F, -G, et -H ont toutes marqué de façon spécifique le récepteur NPR-A monomérique (130 kDa). Au contraire, ^{125}I-pBNP1-E s'est montré incapable de toute incorporation. Les taux maximum d'incorporation ont été mesurés à 18%, 41%, 6%, 25%, 40%, et 40%, pour ^{125}I-pBNP1-A, -B, -C, -F, -G, et -H, respectivement. La digestion du complexe obtenu par le photomarquage du récepteur avec chacune des sondes a révélé deux motifs distincts en SDS-PAGE, avec ^{125}I-pBNP1-A, -B, -C, -G, et -H, affichant une bande majeure à 16 kDa, et des bandes additionnelles à 40, 34, 13, et 4 kDa, alors que le motif obtenu avec pBNP1-F n'a affiché que deux bandes, une majeure à 4 kDa, et une mineure à 40 kDa. La réduction et l'alkylation de ce fragment de 4 kDa, et son analyse subséquente par 16T6C SDS-PAGE a révélé une bande de poids moléculaire supérieure à celle obtenue par la migration de la sonde réduite et alkylée, mais non complexée au récepteur.

Ces résultats suggèrent la présence d'un nouveau point de contact entre les peptides natriurétiques et le récepteur NPR-A. Au niveau du ligand, ils localisent ce second épitope à l'intérieur de la structure cyclique. La synthèse d'une sonde photoactivable bisubstituée, dérivée du pBNP1 et incorporant un groupement benzophénone en C-terminal (Y28Bpa) ainsi qu'à l'intérieur du cycle (L18Bpa), se présente dès lors comme une stratégie intéressante pour simultanément photomarquer ces deux sites distincts. Ces travaux permettraient de déterminer si ces sites de reconnaissance se retrouvent sur la même sous-unité du récepteur homodimérique, ou si au contraire les peptides natriurétiques interagissent simultanément avec les deux sous-unités du récepteur NPR-A.

Photoaffinity scanning of the bovine adrenal zona glomerulosa natriuretic peptide receptor type-A (NPR-A) using benzophenone probes derived from [N,C,rANP(1-28)]pBNP32 (pBNP1)

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List of abbreviations

ANP, atrial natriuretic peptide; Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate; Bpa, *p*-benzoyl-L-phenylalanine; DIPEA, diisopropylethylamine; NPR-A, natriuretic peptide receptor type A; pBNP32, porcine brain natriuretic peptide; pBNP1, [N,C,rANP(1-28)]pBNP32; pBNP1-A, [N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1; pBNP1-B, [N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1; pBNP1-C, [Tyr^{2}, Bpa^{3}]pBNP1; pBNP1-D, [Tyr^{6}, Bpa^{9}]pBNP1; pBNP1-E, [Bpa^{16}, Tyr^{18}]pBNP1; pBNP1-F, [Bpa^{18}, Tyr^{26}]pBNP1; pBNP1-G, [Tyr^{18}, Bpa^{26}]pBNP1; pBNP1-H, [Tyr^{18}, Bpa^{28}]pBNP1.

Abstract

A SAR-directed photoaffinity-scan using benzophenone substituted high affinity chimeric analogs of [N,C,rANP(1-28)]pBNP32 (known as pBNP1) (Mimeault et al., 1993, *Mol. Pharmacol. 43*, 775-782), was carried out in order to further document the binding dynamics of natriuretic peptides toward the homodimeric natriuretic peptide receptor type-A (NPR-A). Using solid phase peptide synthesis, we synthesized the 8 following pBNP1 derived probes: [N^{α}-*p*-benzoylbenzoyl, L2Y]pBNP1 (A); [N^{α}-*p*-benzoylbenzoyl, L18Y]pBNP1 (B); [L2Y, R3Bpa]pBNP1 (C); [S6Y, G9Bpa]pBNP1 (D); [G16Bpa, L18Y]pBNP1 (E); [L18Bpa, F26Y]pBNP1 (F); [L18Y, F26Bpa]pBNP1 (G); and [L18Y, Y28Bpa]pBNP1 (H). Binding affinities were tested on membrane preparations of bovine adrenal *zona glomerulosa* NPR-A. All linear peptides displayed low affinities. Cyclic pBNP1-A, -B, -C, -F, -G, and -H affinities were found equipotent to ANP (Δ pK_i ranging from -0.04 to +0.14), while pBNP1-D (Δ pK_i = -0.43 $\pm$ 0.10) displayed an affinity being 3-fold less. Substitutions in pBNP1-E yielded a peptide 100-fold less potent than ANP (Δ pK_i = -1.76 $\pm$ 0.08), and was therefore rejected as a probe. Iodination by lactoperoxydase kept diiodination minimal while preferentially targeting residue Tyr^{28}. Benzophenone substitutions made in the N-terminal segment (pBNP1-A, -B, -C) yielded the probes with highest non-specific binding (34%, 22%, and 20%, respectively), while substitutions made in the ring (pBNP1-E, -F), and in the exocyclic carboxyl segment (pBNP1-G, -H) yielded probes exhibiting non-specific binding of 15%, 14%, 7%, and 6%, respectively. ^{125}I-pBNP1-A, -B, -C, -F, -G, and -H were all able to specifically cross-link the 130 kDa monomeric NPR-A receptor, contrary to ^{125}I-pBNP1-E which did not incorporate at all. Maximal incorporation yields were established at 18%, 41%, 6%, 25%, 40%, and 40%, for ^{125}I-pBNP1-A, -B, -C, -F, -G, and -H, respectively. Cyanogen bromide digestion of the photolabeled receptor yielded two distinct patterns, with ^{125}I-pBNP1-A, -B, -C, -G, and -H, all displaying a major fragment at 16 kDa, and additional bands at 40, 34, 13, and 4 kDa. Interestingly, pBNP1-F displayed a unique pattern, with only two bands, one at 40kDa, and a major one at 4 kDa. Reduction and alkylation of this tagged 4 kDa fragment, and its subsequent analysis by 16T6C SDS-PAGE, yielded a band of superior molecular weight than the reduced and alkylated labeled pBNP1-F. These results suggest that both the amino and carboxyl terminal ends interact with

similar regions of the receptor binding pocket, while the peptide loop binds to a distinct region of the NPR-A receptor.

Introduction

Atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) exert their cardiovascular homeostatic actions through natriuretic and diuretic effects following their specific binding to a particulate guanylyl cyclase known as the natriuretic peptide receptor type-A (NPR-A) (Anand-Srivastava and Trachte, 1993; Lewicki and Protter, 1995; McDowell et al., 1995). As a member of the guanylyl cyclase-linked receptor family (Yuen and Garbers, 1992), NPR-A is characterized by a large extracellular binding domain, a short membrane spanning domain, and a cytoplasmic domain divided in two subdomains: a regulatory domain baring homologies with the protein tyrosine kinases, and a catalytic domain displaying high homologies with soluble guanylyl cyclase. Signaling involves 1) high affinity ligand binding to the noncovalent homodimer receptor (Rondeau et al., 1995; Meloche et al., 1987; Meloche et al., 1988); 2) modification of the homodimer conformation (Liu et al., 1989; Ullrich and Schlessinger, 1990; Milburn et al., 1991; Milligan et al., 1991); 3) concomitant availability of an ATP regulatory module (ARM) in the protein tyrosine kinase homology domain (Goraczniak et al., 1992; Marala and Sharma, 1992); and 4) ATP binding which diminishes the ligand affinity (Larose et al., 1991; De Léan 1986), and more importantly, inhibits the constitutive down-regulation of the receptor catalytic moiety responsible for cellular accumulation of the second messenger guanosine cyclic 3' 5' monophosphate (Leitman et al., 1987; Chinkers and Garbers, 1989). Nevertheless, and despite these established facts, the exact transduction mechanism remains controversial. In that view, understanding the binding dynamics is crucial.

NPR-A recognition site has been previously investigated. Microsequencing of a labeled chymotryptic fragment obtained by UV affinity cross-linking of ^{125}I-[Tyr^{2}]rANP(2-27) with affinity purified NPR-A yielded the glycosylated segment Met^{173} - Phe^{188} (McNicoll et al., 1996). Overlapping of labeled proteolytic fragments of bovine adrenal zona glomerulosa NPR-A photolabeled with the high affinity probe [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (McNicoll et al., 1992) targeted the neighboring region Asp^{191} - Arg^{198} (McNicoll et al., 1996). Both sequences are highly variable (Féthière & De Léan, 1991; Schoenfeld et al., 1995) suggesting species specificity, while glycosylation of Asn^{180} appears important for surface availability (Liu et al., 1989; McNicoll et al., 1996). Furthermore, use of the probe [*p*-azidobenzoyl-Ser^{1}, Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-27) (Rondeau et al., 1995) resulted in the identification on SDS-PAGE of a

protein M_r 250 kDa corresponding to the homodimer. It is now well established that several receptors of the protein-tyrosine kinase family undergo homodimerization upon activation Such is the case for the receptors of the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF), the latter hormone being itself dimeric, and therefore containing two receptor binding epitopes, each one interacting with a distinct subunit (Heldin, 1995). Such is also the case for the erythropoietin binding protein (EBO), an erythropoietin (EPO) peptide agonist. Upon resolution of the crystal complex of EBO with the extracellular domain of the EPO receptor, it was determined that EBO homodimerizes and interact with the homodimeric receptor through two binding epitopes, one on each subunit (Livnah et al., 1996). In the case of the human growth hormone binding protein (hGHbp), it was found to homodimerize in a sequence where one hGH binds one receptor subunit, which then drives the formation of the homodimeric receptor complex by interacting with the second subunit. (De Vos et al., 1992). Noteworthy is the fact that while both receptors contribute the same residues, both epitopes on the hormone share no structural similarity. Those examples not only stress the validity of the dimeric model as a viable transduction mechanism, but also underline the high variability between receptors of the process itself. Despite the established homologies between NPR-A and the protein kinase receptor family, it is not yet known if both NPR-A subunits delineate the ligand binding domain, and if so, if the ligand interacts with both, as suggested with [*p*-azidobenzoyl-Ser^{1}, Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-28).

Thus, we further investigated the ligand/receptor interaction by undertaking a photoaffinity-scan using synthetic analogs of benzophenone-derived [N,C,rANP(1-28)]pBNP32 (known as pBNP1) (Mimeault et al., 1993) (Figure 1). Choice of this chimeric peptide as a synthesis template was based on its high affinity for bovine NPR-A (pK_i = 11.26), a strategy aiming at counteracting the potential negative substitution effects and therefore maximizing the affinity of the photoprobes. SARS and NMR analyses of natriuretic peptides and various analogs oriented our molecular mapping strategy, thus precluding the systematic Bpa scanning approach.

General agreement is that ANP N-terminal segment is of moderate importance for binding (Bovy, 1990), whereas the intraloop segment Cys^{7} - Gly^{16}, and the C-terminal segment Phe^{26} - Arg^{27} are crucial for receptor binding and activation (Inooka et al., 1990; Kobayashi et al., 1988). As a matter of fact, the ligand binding dependent residues, determined by SARS done mainly on rat

and human ANP, and identified as Phe^{8}, Ile^{12}, (Met^{12}), Asp^{13}, Arg^{14}, Ile^{15}, Leu^{21}, and Arg^{27}, (Nutt et al., 1988; Cunningham et al., 1989; Bovy, 1990) are exclusively located in the hormone ring portion and C-terminal segment. Nonetheless, removal of Arg^{3} and Arg^{4} from rANP was found to produce a 10-fold loss in affinity toward bovine adrenal zona glomerulosa NPR-A (De Léan et al., 1985). Several NMR analyses of natriuretic peptides or related analogs have underline the presence of a hydrophobic cluster formed by the side-chain of the amino acids Phe^{8}, Ile^{12}, Ile^{15}, and Leu^{21} (pBNP1/phosphatidylcholine) (Carpenter et al., 1997), Phe^{8}, Ile^{12}, Ile^{15}, Leu^{21}, and Phe^{26} (hexamutant ANP/DMSO) (Fairbrother et al., 1994), or Phe^{8}, Ile^{15}, Leu^{21} (mini-ANP/DMSO) (Li et al., 1995).

Baring in mind possible interactions between the hormone cyclic core and the receptor, our scan addressed all three segments of the synthetic peptide, with pBNP1-A, -B, and -C incorporating the photophore in the N-terminal segment, pBNP1-D, -E, and -F targeting the ring structure, and pBNP1-G, and -H pinpointing the exocyclic carboxyl segment. Both pBNP1-A and -B were designed to display a N^{α} benzophenone moiety. Additions were achieved through the use of p-benzoylbenzoic acid and BOP as the coupling reagent. In all other cases, ponctual substitutions with *p*-benzoyl-L-phenylalanine (Bpa) were made in the immediate viscinity of crucial residues, with pBNP1-C (R3Bpa), -D (G9Bpa), and -E (G16Bpa), being positionned next to Arg^{4}, Phe^{8}, and Ile^{15}, respectively, while pBNP1-F (L18Bpa) was located between Ile^{15} and Leu^{21}. pBNP1-G (F26Bpa), and -H (Y28Bpa) were located as to flank Arg^{27}. Tyrosine substituted residues were chosen on their hydrophobic nature, and iodination accessibility, based on pBNP1 NMR generated models (Carpenter et al., 1997).

Experimental procedures

Materials for peptide synthesis. Boc-Arg(Tos)-OH, Boc-Ser(OBzl)-OH, Boc-Cys(Acm)-OH and Boc-Leu-OH•H_2O were from Bachem inc. (Torrance, CA). Boc-Phe-OH, Boc-Asp(cHex)-OH were from Propeptide. Boc-Tyr(BrZ)-OH and Bpa were from Advanced Chemtech. Boc-Gly-OH, Boc-Ile-OH•½H_2O, and *p*-benzoylbenzoic acid were from Sigma Canada Ltd. (Mississauga, Ontario, Canada). BOP was from Richelieu Biotechnologies (Montréal, Québec, Canada). ACS-grade dimethylformamide and methylene chloride were from Anachemia Canada inc. (Ville St-Pierre, Québec, Canada). Diisopropylethylamine and cesium hydrogen carbonate were from Aldrich (Milwaukee, WI). Chloromethylated resin (copolystyrene-1% divinylbenzene, 0.75 meq/g) was from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario, Canada). Biograde trifluoroacetic acid was from Halocarbon (Hackensack, NJ).

Materials for peptide characterization and photoaffinity labeling. Bovine kidneys were obtained from a local slaughterhouse. Aprotinin, leupeptin, pepstatin A, phenylmethanesulfonyl fluoride (PMSF) were from Sigma Canada Ltd. (Mississauga, Ontario, Canada). Carrier-free $Na^{125}I$ was from Amersham Life Science Inc. (Arlington Heights IL). Bovine serum albumin, carboxypeptidase A, sodium dodecyl sulfate, tris(hydroxymethyl)aminomethane, and glycine were from Boehringer Mannheim Canada (Laval, Québec, Canada). Electrophoresis reagents and standards were from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario, Canada). GF/C filters were obtained from Fisher Scientific Canada (Montréal, Québec, Canada). Poly(ethyleneimine), and tricine were from Aldrich (Milwaukee, WI).

Peptide synthesis. All peptides were synthesized by solid-phase peptide synthesis (Merrifield, 1964) using a homemade manual multireactor system. Chloromethylated resin served as solid support. Couplings were carried out with 3 eq. of BOP reagent, 3 eq. of Boc-AA-OH and 5 eq. of diisopropylethylamine. Monitoring of residue addition was achieved through the ninhydrin test (Kaizer et al., 1970). Amino acid side-chain deprotection, as well as peptide cleavage from the solid support, were achieved through use of liquid hydrogen fluoride.

Peptide purification. All crude analogs were purified by preparative reverse-phase HPLC on a Waters Prep LC 500A system equipped with a Waters 1000 Prep Pak Module and a 441

absorbance detector. Peptide solutions (700 mg in H_2O (0.06% trifluoroacetic acid) were injected on a DeltaPak C_{18} (15 μm, 30 nm) reverse-phase column (30 cm x 4.7 cm). The material was eluted with a linear gradient of (A) H_2O (0.06% trifluoroacetic acid) and (B) acetonitrile (35%) in H_2O, as follows: 0% of B to 100% B in 90 min. Flow rate was set at 60 ml/min and detection was performed at 230 nm. Individual fractions 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} (10 μm) reverse-phase column (30 cm x 0.39 cm) and an eluate of (A) H_2O (0.06% trifluoroacetic acid) and (B) acetonitrile in a linear gradient mode (15% B to 45% B in 15 min). Flow rate was set at 1.5 ml/min and detection was performed at 230 nm. Additional analyses were made using a Pharmacia Super Pak C_{18} (5μm) reverse-phase column (25 cm x 0.4 cm) and an eluate of (A) H_2O (0.06% trifluoroacetic acid) and (B) acetonitrile in a linear gradient mode (20% B to 40% B in 15 min) at a flow rate of 1 ml/min. Detection was performed at 230 nm.

Preparation of membranes. Bovine adrenal *zona glomerulosa* membranes were prepared as previously described (Meloche et al., 1986). 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 membranes 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-rANF(99-126) 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-rANF(99-126) 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 an 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 rANF(99-126). Experimental conditions were the same as those described above.

Iodination of analogs. 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 $Na^{125}I$ 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 enzyme was extracted by filtration on a 0.45 μ nylon 66 Conz Syringe-Tip filter, and the iodinated peptide purified on a Vydac C_{18} (10μm) 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. At the end of a 30 min incubation at 37°C, the reaction mixture was filtered through a Waters C_8 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 using treated ^{125}I-pBNP1 as a control.

Photoaffinity labeling with iodinated analogs. Radioligand binding was achieved as stated above. At the end of the incubation period the scavenger *p*-aminobenzoic acid was added (10^{-7} M) to the binding mixture. Tubes were sparged 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 time irradiation. Tubes were then centrifuged at 40000 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 then 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.

Photolabeled receptor digestions. The photoaffinity labeled receptor was purified by preparative electrophoresis using a 3 mm thick 7.5% SDS-PAGE (Laemmli, 1970). Bands corresponding to 130 kDa proteins were sliced into 1 mm x 1 cm fragments and eluted 24 h in ten volumes of 100 mM ammonium bicarbonate buffer. Eluant was filtered and freeze-dried. Prior to cleavage at methionine residues, the complex was reduced under nitrogen through a 4 h incubation with 5% (v/v) 2-mercaptoethanol in 50 mM ammonium bicarbonate buffer, pH 8.0, and then freeze-dried. CNBr cleavage was performed in 70% formic acid for 24 h at ambiant temperature (CNBr : protein, 1:1, w/w). The digestion medium was diluted 10-fold with water, and the reaction mixture freeze-dried. Recovered proteins were then dissolved in sample buffer and submitted to 16T6C SDS-PAGE (Laemmli, 1970). Proteins were finally eluted from gel and transfered on a nitrocellulose support before beeing revelated using a phosphor screen from Molecular Dynamics.

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 (De Léan et al., 1982; De Léan et al., 1978).

Results

Peptide synthesis and characterization. All peptides (Figure 1) were made on chloromethylated resin by a Boc/BOP stepwise solid phase peptide synthesis approach using a homemade multireactor system (Merrifield, 1964; Castro et al., 1975; Fournier et al., 1988). Both *p*-benzoylbenzoyl-N^{α} derivatization were achieved in situ with *p*-benzoylbenzoic acid and BOP as the coupling reagent (Coupal et al., 1998 *paper under review*). In all other cases, the benzophenone moiety was introduced in the form of the modified amino acid *p*-benzoyl-L-phenylalanine. Purification of the linear analogs was achieved through preparative HPLC, and purity was assessed by analytical HPLC (≥98%). Purification of the cyclic analogs was also achieved through preparative HPLC. As compared to pBNP1 which elutes on a C_{18} reverse-phase column at 33 % of acetonitrile, synthetic peptides pBNP1-A, -B, -C, -D, -E, -F, -G, and -H eluted at 37%, 38%, 36%, 38%, 37%, 34%, 35%, and 36%, respectively, showing peptides with enhanced hydrophobicity. Purity assessment was adressed by analytical HPLC (≥98%), capillary electrophoresis, and mass spectroscopy (expected m/z for pBNP1-A: 3420.9; -B: 3420.9; -C: 3307.8; -D: 3433.0; -E: 3406.9; -F: 3316.8; -G: 3316.8; and -H: 3300.8, respectively; obtained: -A: 3421.2 ± 0.5; -B: 3421.5 ± 0.6; -C: 3308.4 ± 0.6; -D: 3433.7 ± 0.8; -E: 3405; -F: 3317.6 ± 0.7; -G: 3318.1; and -H: 3301.8, respectively).

NPR-A affinity toward synthesized analogs. All peptides were tested for affinity by competitive binding assays against ^{125}I-rANP(1-28) on membrane preparations of bovine adrenal *zona glomerulosa* NPR-A (De Léan et al., 1984; Meloche et al., 1986; Meloche et al., 1988) (Figure 2). As expected, all linear analogs displayed very poor affinity, being about 100-fold less potent then their cyclic counterpart (data not shown), stressing the importance of the disulfide link (Bovy, 1990). In the case of cyclic analogs, pBNP1-D distinguished itself by its poor affinity (ΔpK_i = -1.76 ± 0.08), being about 50-fold less potent then ANP. Also less potent, pBNP1-E (ΔpK_i = -0.43 ± 0.10), which displayed an affinity being about 3-fold lower than ANP. As for pBNP1-A, -B, -C, -F, -G and -H, ΔpK_i were measured at 0.11 ± 0.11, 0.00 ± 0.11, 0.08 ± 0.19, 0.14 ± 0.11, -0.04 ± 0.08, and 0.03 ± 0.11, respectively, confirming that substitutions made translated into only little loss of affinity as compared to their pBNP1 template (pK_i = 11.26). Furthermore, all six peptides displayed ANP-type high affinities (pK_i = 10.71) (Figure 2).

Peptide iodination. Despite the presence of two tyrosines in 7 out of the 8 analogs synthesized, diiodination was kept minimal (data not shown), producing labeled probes with a specific activity of 2000 Ci/mmol. Indeed, highly preferential targeting by the enzyme of the Tyr^{28} residue was observed. Treatment with carboxypeptidase A of the analogs ^{125}I-pBNP1-A (Tyr^{2}), ^{125}I-pBNP1-D (Tyr^{6}), and ^{125}I-pBNP1-B (Tyr^{18}), resulted in a loss of radioactive signal in the proportions of 80%, 75%, and 90%, respectively. In the case of ^{125}I-pBNP1-F (Tyr^{26}), the treatment resulted in the loss of only 56% of the signal, the enzyme unpreferentially targeting either tyrosine (Table 1). In all cases, the radiolabeled probes eluted at a 2% higher concentration of acetonitrile, assuring segregation from the unlabeled peptides.

Specific binding. Introduction of the benzophenone moiety yielded in several cases peptides with enhanced hydrophobicity. This was translated by high non-specific binding, especially in the case of ^{125}I-pBNP1-A, with 34% of non-specific binding as computed by saturation curve analysis. Percentages for ^{125}I-pBNP1-B, -C, -E, and -F, were established at 22%, 20%, 15%, and 14%, respectively, while both ^{125}I-pBNP1-G, and -H non-specific bindings were significantly lower with percentages measured at 7%, and 6%, respectively.

Photoaffinity labeling. The probes ^{125}I-pBNP1-A, -B, -C, -E, -F, -G, and -H were tested for covalent incorporation into the NPR-A receptor binding domain by UV activation at 365 nm. The process was carried out in the absence of oxygen. Nevertheless, non-specific incorporation due to lipid peroxydation (Larose et al., 1990) was monitored with ^{125}I-pBNP1 as a control. Photoaffinity labeling was documented by autoradiography following SDS-PAGE. It showed that ^{125}I-pBNP1-A, -B, -C, -F, -G, and -H were all able to specifically cross-link the 130 kDa monomeric NPR-A receptor. Interestingly, ^{125}I-pBNP1-E, while displaying high affinity, did not specifically cross-link the receptor (Figure 3). Maximal incorporation yields, as the percentage of the reversibly bound ligand after correction for the lipid peroxydation-driven non-specific cross-linking (2-3%), were set at 6%, 25%, 40%, and 40%, for ^{125}I-pBNP1-C, -F, -G, and -H, respectively, while pBNP1-A, and -B maximal incorporation yields were set at 18%, and 41%, respectively (Table 1). Due to its low affinity, pBNP1-D was not used as a probe.

Photolabeled NPR-A digestions. Once photoaffinity labeled with the probes ^{125}I-pBNP1-A, -B, -C, -G, and -H, digestion of the cross-linked receptor with CNBr followed by SDS-PAGE produced the same pattern in all cases, with a major fragment at 16 kDa and additionnal bands at 40, 34, 13, and 4 kDa (Figure 4). Interestingly, the same procedure with the probe ^{125}I-pBNP1-F yielded a different pattern, with the presence of only two bands, one at 40 kDa, and a major one at ≈4 kDa. In order to better characterize this small fragment relative to free ligand, it was eluted from the gel, reduced and alkylated, and then submitted to a 16T6C SDS-PAGE in parrallel with reduced and alkylated free ^{125}I-pBNP1-F (Figure 5). Results underline the larger mass of the tagged fragment as compared to the free labeled probe and confirm that the 4 kDa band corresponds to a small receptor fragment covalently bound to the peptide ligand.

Discussion

Benzophenone (BP) photochemistry was shown in numerous studies to be a highly efficient site-specific cross-linking tool for site mapping (Dorman, and Prestwich, 1994; Prestwich et al., 1997 *for reviews*). BP low reactivity toward water molecules, combined with its strict geometrical requirements, ensured superior photochemical behaviors then those of aryl azides, aryl diazirines, alpha-diazocarbonyls, or lipid peroxydation cross-linking (Weber and Beck-Sickinger, 1997; Pilch & Czech, 1983; Larose et al, 1990). Furthermore, choice of pBNP1 ([N,C,rANP(1-28)]pBNP32) (Mimeault et al., 1993) as the synthetic template was made in order to maximize the probe affinity. Pharmacological characterization using binding assays on bovine adrenal *zona glomerulosa* NPR-A confirmed an ANF-type high affinity for pBNP1-A, -B, -C, -F, -G, and -H, with ΔpK_i ranging from -0.04 to 0.11. Substitutions made in these probes translated into only little losses of affinity as compared to pBNP1. In fact, the 3-fold loss was compensated by the 3-fold gain provided by the chimeric peptide, establishing pBNP1 as a valid template. While still an efficient probe, pBNP1-E displayed a 3-fold lower affinity ($\Delta pK_i = -0.43 \pm 0.10$). On the other hand, the pBNP1-D binding results showed an important loss of affinity, possibly related to a loss of structural integrity ($\Delta pK_i = -1.76 \pm 0.08$). Indeed, an explanation may reside in the fact that the BPA^9 and / or Tyr^6 side-chains are in proximity of Cys^7, thus creating steric hindrance, which could in turn affect orientation of the disulfide bond. Such structural stresses were reported with associated loss of affinity and potency (Koide et al., 1993). It has also been suggested that the disulfide bridge itself may interact directly with the receptor recognition site (Bovy, 1990).

With the exception of pBNP1-E, found unable to cross-link the receptor, probes displaying ANP-type affinities were all able to specifically photolabel a protein of M_r 130 kDa (Fig. 3), corresponding to the NPR-A receptor monomer. All gels displayed clean profiles, again validating adrenal *zona glomerulosa* membrane preparations as a characterizing model. While high incorporation yields (›70%) have been reported with the use of benzophenone for photolabeling (McNicoll et al., 1992; Dorman & Prestwich, 1994), we report incorporation efficiencies ranging from 6% to 41%. Such low yields (2% - 5%) have also been reported in the case of high affinity benzophenone derivatives of SP (Kasheverov et al., 1997) and vasopressin (Kojro et al., 1993), underlining variability of the benzophenone photophore efficiency. Worth

noting, photoinduced cross-linking yields of [^{125}I]-pBNP1-A, -B, -C, -F, -G, and -H, differed substantially depending on the photophore positioning, with specific incorporation percentages measured at 18%, 41%, 6%, 25%, 40%, and 40%, respectively. Again, such a behavior was also reported in the case of Substance P derivatives with incorporation yields of 46% for Bpa^{8}-SP as compared to 6% for Bpa^{3}-SP (Boyd et al., 1996; Li et al., 1995; Girault et al., 1996).

Bearing in mind the restrictive cross-linking prerequisites of the benzophenone photophore, which limit to 3Å the distance between the reactive species (Dorman & Prestwich, 1994), as well as the fact that pBNP1-A, -B, -C, -F, -G, and -H, all display ANP-type affinity potencies (Table 1), it becomes plausible to view the incorporation yields as a function of the benzophenone distance and orientation once the ligand is linked to its receptor. Based on such an approach, our results would be in agreement with molecular models of natriuretic peptide and various analogs, which describe the C-terminal segment and the cyclic core close in space and interacting with the receptor recognition site (Bovy, 1990; Carpenter et al., 1997; Fairbrother et al., 1994). In addition, this postulate would be in agreement with the model of [Tyr^{18}, Bpa^{27}]ANP(1-27) derived by molecular modeling using internuclear distance constraints obtained with NMR studies of ANP (McNicoll et al., 1992). In this model both Bpa^{27} and Tyr^{18} residues are forming a hydrophobic core which would interact with the NPR-A binding pocket. In fact, both probes [^{125}I]-pBNP1-G, and -H, displayed high incorporation yields (40% for both), while [^{125}I]-pBNP1-F (25%) appeared as the only probe derivatized in the ring portion still able to cross-link the receptor. It is worth mentioning that [^{125}I]-pBNP1-E (0%) has been unable to cross-link the receptor despite an excellent affinity ($\Delta pK_i = -0.43 \pm 0.10$).

Non-specific binding (N), corresponding to the ratio of bound ligand versus free ligand when assessed with saturating concentrations of rANP (10^{-7} M) was found important, especially in the cases of [^{125}I]-pBNP1-A, -B, and -C, with N reaching 33%, 22%, and 21%, respectively. Such drawbacks have also been reported in the case of Substance P (SP) derivatives baring a benzophenone photophore in the N-terminal segment of the peptide (Kasheverov et al., 1997). As a matter of fact, the probe [^{125}I]-N$^{\alpha}$-(p-benzoylbenzoyl), N-(-Lys 3-(p-hydroxyphenylpropionyl)-SP displayed a non-specific binding background reaching 80% of the

total binding. Interestingly, the analogs N-(-Lys 3-(p-hydroxyphenylpropionyl)-SP (Kasheverov et al., 1997) and Bpa^3-SP (Li et al., 1995) did not yield probes with enhanced non-specific binding, suggesting that positioning the benzophenone moiety at the end of a spacer such as the lysine side-chain is not suitable. In that view it is worth mentioning that NMR studies underl ned the random coil nature of the N-terminal segment of the natriuretic peptide (Carpenter al al., 1997; Fairbrother et al., 1994). This N-terminal stretch moving freely is similar to a flexible spacer and favors randomized hydrophobicity-driven interactions. Such a behavior would be in agreement with the fact that pBNP1-A, which exhibits a benzophenone moiety immediately flanked with a tyrosine residue, displayed higher non-specific binding than pBNP1-B. Moreover, Bpa substitutions made in the more structured C-terminal segment ([^{125}I]-pBNP1-G, and -H) yielded probes with N values measured at 6%, and 7% respectively, thus being comparable to the 2% displayed by the probe [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANF(1-27) (McNicoll et al., 1992). As for [^{125}I]-pBNP1-F, the N value was set at 14% and viewed as intermediate.

Once cross-linked to the receptor, all functional probes were submitted to CNBr digestion (Fig. 4). The synthetic derivatives pBNP1-A, -B, -C, -G, and -H displayed the same pattern on SDS-PAGE, with a major band at 16 kDa, and minor bands at 40, 34, 13, and 4 kDa. These results are consistant with the CNBr digestion pattern of NPR-A when affinity cross-linked with [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-28) and displaying a major band at 15 kDa, with presence of minor bands at 40, 32, and 12 kDa (McNicoll et al., 1992). Surprisingly, pBNP1-F displayed a unique pattern consisting of a major band at 4 kDa, and a minor one at 40 kDa, with total absence of intermediary bands at 34, 16, and 13 kDa. This original pattern was found to be highly reproducible (n=3). Furthermore, controls showed that treatment of the complex with 70% formic acid was not the source of such a thourough digestion (data not shown). Further investigation was achieved through gel elution of this 4 kDa band, followed by protein reduction with DTT and alkylation with iodoacetic acid, before submitting the material to a 16T6C SDS-PAGE analysis (Schägger et al., 1987) in presence of reduced and alkylated ^{125}I-pBNP1-F (Fig. 5). While only qualitative, results showed that the labeled fragment is of a larger mass than the free labeled ligand, suggesting that pBNP1-F is cross-linked to a small CNBr-generated fragment. The fact that pBNP1-F displayed a different pattern than those of pBNP1-G and -H was

somewhat surprising, as we always considered Tyr^{18} and Bpa^{27} of [Tyr^{18}, *p*-benzoyl-Phe^{27}]ANP(1-28) (McNicoll et al., 1992) as forming a coherent hydrophobic core, a model supported by numerous structural NMR studies viewing the C-terminal segment in a kink pattern toward the peptide ring (Carpenter et al., 1997; Fairbrother et al., 1994). It is worth underlining that the bovine NPR-A sequence is still not fully elucidated although our laboratory has now microsequenced 20% of the receptor protein (McNicoll et al., 1996; Rondeau et al., 1992). In that view, our results may indicate the presence of a still unknown methionine residue which would be responsible for generating such a small fragment. Also possible, and caused by the presence of formic acid (Tarr and Crabb, 1983), might be the partial cleavage of a Asp-Pro bond in the vicinity of a methionine residue. While possible, these interpretations are not in agreement with the fact that the partial bovine NPR-A sequence obtained so far displays a 97% homology with human NPR-A, the latter having been entirely sequenced (Schoenfeld et al., 1995), and presenting none of the above residues in the vicinity of Met^{173} - Phe^{188} or the neighboring region Asp^{191} - Arg^{198}. Interestingly, sequence overlapping of human, rat, and mouse NPR-A displays three different residues at position 228 (E, N, and D, respectively) (Schoenfeld et al., 1995), emphasizing the possibility of a Met^{228} residue in the bovine receptor. In such a case, pBNP1-F, after cross-linking the hypothetical segment Met^{223} - Met^{228}, flanking the important region Met^{173} - Met^{223}, could indeed generate a very small fragment. Of course, and worth stating, is the postulated model involving two subunits of the receptor as delineating the binding pocket, and furthermore, both interacting with the ligand (Rondeau et al., 1995). This was seen with the human growth hormone receptor (Cunningham et al., 1991), also a receptor displaying a single transmembrane domain. In such a case, it can be hypothesized that the natriuretic hormone exocyclic segments could interact with one receptor subunit while the cyclic core would bind to the other.

Our studies have pinpointed the advantages of using pBNP1 as a template for the synthesis of high affinity photolabeling probes. By adressing all three segments of pBNP1, we have developped a family of six potent probes scanning the entire length of the hormone. Furthermore, we have obtained results suggesting that the cyclic core of the probe recognizes a region of the receptor being distinct from that with which interact the exocyclic segments.

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

Fig. 2. Competition and saturation analysis of the pBNP1-F analog.

NPR-A from bovine *zona glomerulosa* membrane preparations (20µg) was incubated 90 min at room temperature with (**A**) approximately 10 pM of ^{125}I-rANP(1-28) and increasing concentrations of unlabeled rANP (■)or pBNP1-F (▲); or (**B**) with increasing concentrations of ^{125}I-pBNP1-F in presence (▲) or absence (■) of unlabeled rANP(1-28) (10^{-6}M). Radioligand binding measured after separation and washing on GF/C filters is reported. Curves were analyzed by the computer program Graphpad.

Fig. 3. NPR-A covalent photoaffinity labeling with ^{125}I-pBNP1-A, -B, -C, -E, -F, -G, and -H.

Bovine adrenal *zona glomerulosa* membrane protein (20 µg/ml) was incubated with each of the labeled probes as to obtain in each case approximately 10000 cpm of specific binding per 20 µg of protein, 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, then centrifuged, the pellet 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.

Fig. 4. CNBr digestion of the receptor cross-linked with the probes ^{125}I-pBNP1-A, -B, -C, -F, -G, and -H.

In each cases (n=3), 3 mg of protein were incubated for 90 min with saturating concentrations of the labeled probes, then submitted to UV induced cross-linking before beeing purified by preparative electrophoresis using a 3 mm thick 7.5% SDS-PAGE (Laemmli, 1970). Bands corresponding to 130 kDa proteins were sliced into thin fragments and eluted 24 h in ten volumes of 100 mM ammonium bicarbonate buffer. Eluant was filtered and freeze-dried. Prior to cleavage at methionine residues, the complex was reduced under nitrogen through a 4 h

incubation with 5% (v/v) 2-mercaptoethanol in 50 mM ammonium bicarbonate buffer, pH 8.0, and then freeze-dried. CNBr cleavage was performed in 70% formic acid for 24 h at ambiant temperature (CNBr : protein, 1:1, w/w). The digestion medium was diluted 10-fold with water, and the reaction mixture freeze-dried. Recovered proteins were then dissolved in sample buffer and submitted to 16% SDS-PAGE (Laemmli, 1970). Protein were finally eluted from gel and transfered on a nitrocellulose support before being submitted to phosphorimagery.

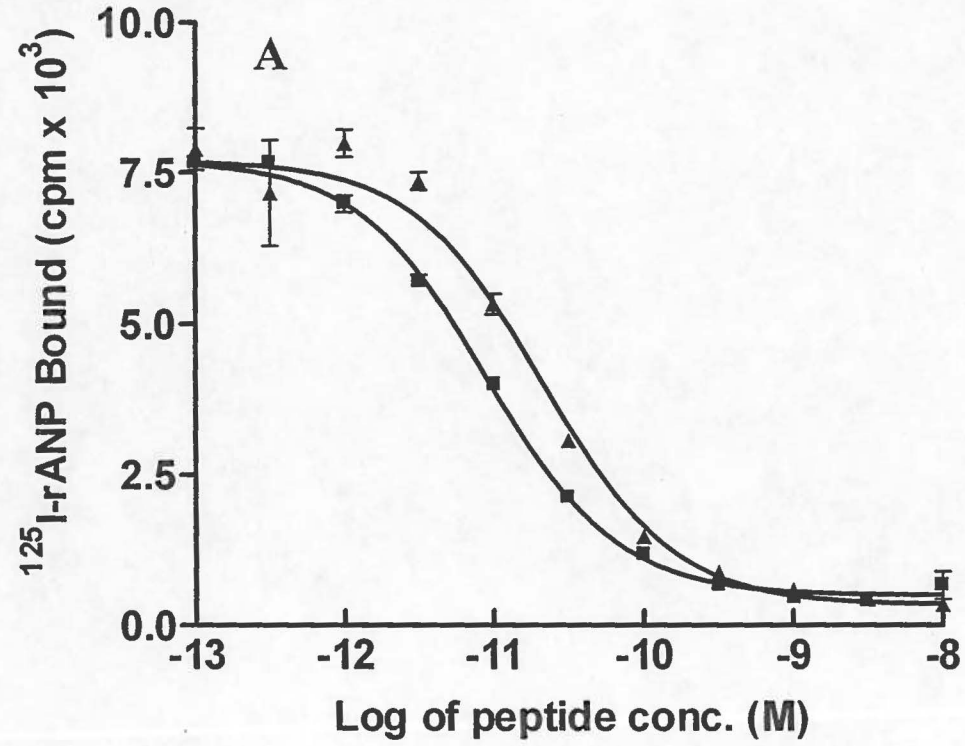
Fig. 5. SDS-PAGE of reduced and alkylated 4 kDa fragment of the receptor complexed with ^{125}I-pBNP1-F.

Following CNBr digestion, the SDS-PAGE band corresponding to the 4 kDa ^{125}I-pBNP1-F cross-linked protein was sliced into thin fragments and eluted 24 h in ten volumes of 100 mM ammonium bicarbonate buffer. Eluant was filtered, freeze-dried, resuspended in a 50 mM ammonium bicarbonate buffer, and than reduced by a 90 min incubation with dithiothreitol (5mM) under nitrogen. Alkylation was performed at room temperature by a 60 min incubation of proteins with 10 mM of iodoacetic acid under nitrogen. Proteins were than submitted to a 16T6C SDS-PAGE analysis. Proteins were eluted from gel and transfered on a nitrocellulose support which was submitted to phosphorimagery analysis. 1) Complex and alkylated ligand; 2) Complex only; 3) Unalkylated ligand and alkylated ligand; 4) alkylated ligand; 5) Unalkylated ligand.

Figure 1

Peptide	Name	Structure
rANP(1-28)	ANP	SLRRSSCFGGRIDRIGAQSGLGCNSFRY
pBNP32	BNP	SPKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
pBNP1	pBNP1	SLRRSSCFGRRLDRIGSLSGLGCNSFRY
[N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1	pBNP1-A	*p*-benzoylbenzoyl-SYRRSSCFGRRLDRIGSLSGLGCNSFRY
[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1	pBNP1-B	*p*-benzoylbenzoyl-SLRRSSCFGRRLDRIGSYSGLGCNSFRY
[Tyr^{2}, Bpa^{3}]pBNP1	pBNP1-C	SYBRSSCFGRRLDRIGSLSGLGCNSFRY
[Tyr^{6}, Bpa^{9}]pBNP1	pBNP1-D	SLRRSYCFBRRLDRIGSLSGLGCNSFRY
[Bpa^{16}, Tyr^{18}]pBNP1	pBNP1-E	SLRRSSCFGRRLDRIBSYSGLGCNSFRY
[Bpa^{18}, Tyr^{26}]pBNP1	pBNP1-F	SLRRSSCFGRRLDRIGSBSGLGCNSYRY
[Tyr^{18}, Bpa^{26}]pBNP1	pBNP1-G	SLRRSSCFGRRLDRIGSYSGLGCNSBRY
[Tyr^{18}, Bpa^{28}]pBNP1	pBNP1-H	SLRRSSCFGRRLDRIGSYSGLGCNSFRB

Figure 2


A
10.0
7.5
5.0
2.5
0.0
-13
-12
-11
-10
-9
-8
^{125}I-rANP Bound (cpm x 10^3)
Log of peptide conc. (M)

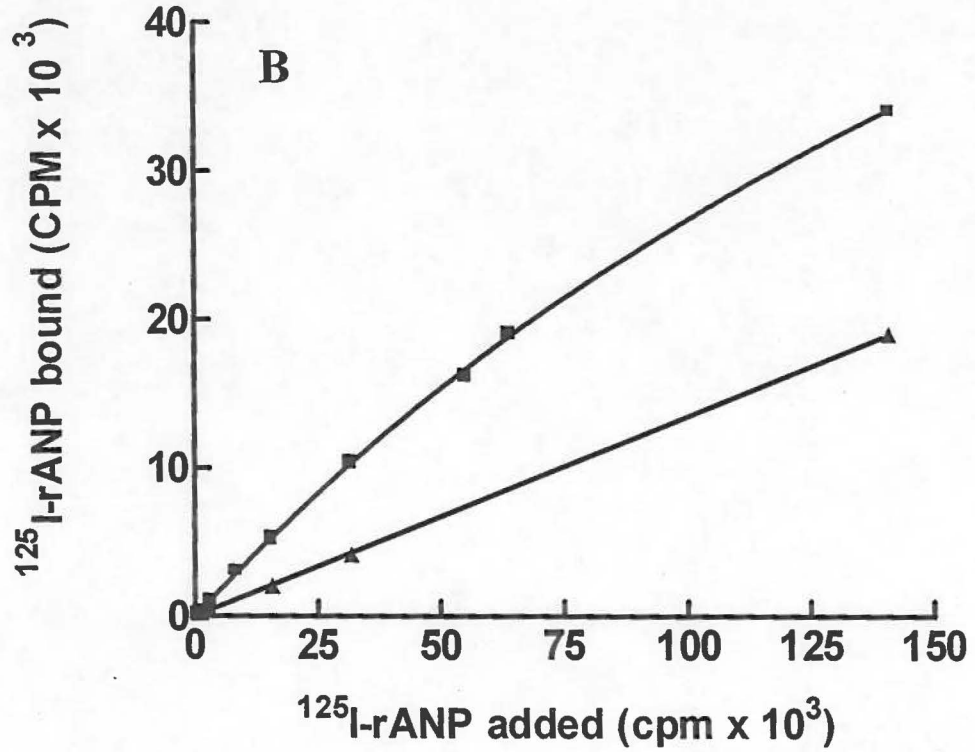

B
40
30
20
10
0
0
25
50
75
100
125
150
^{125}I-rANP bound (CPM x 10^3)
^{125}I-rANP added (cpm x 10^3)

Figure 3

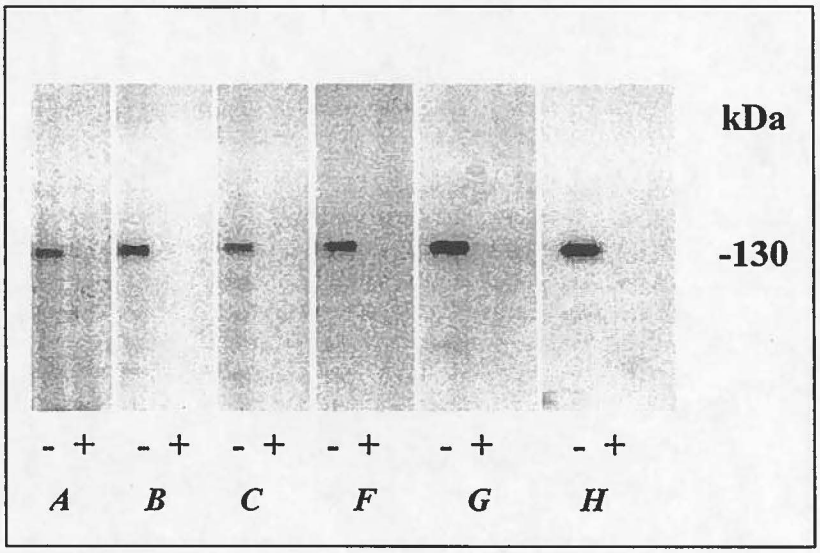


Figure 4

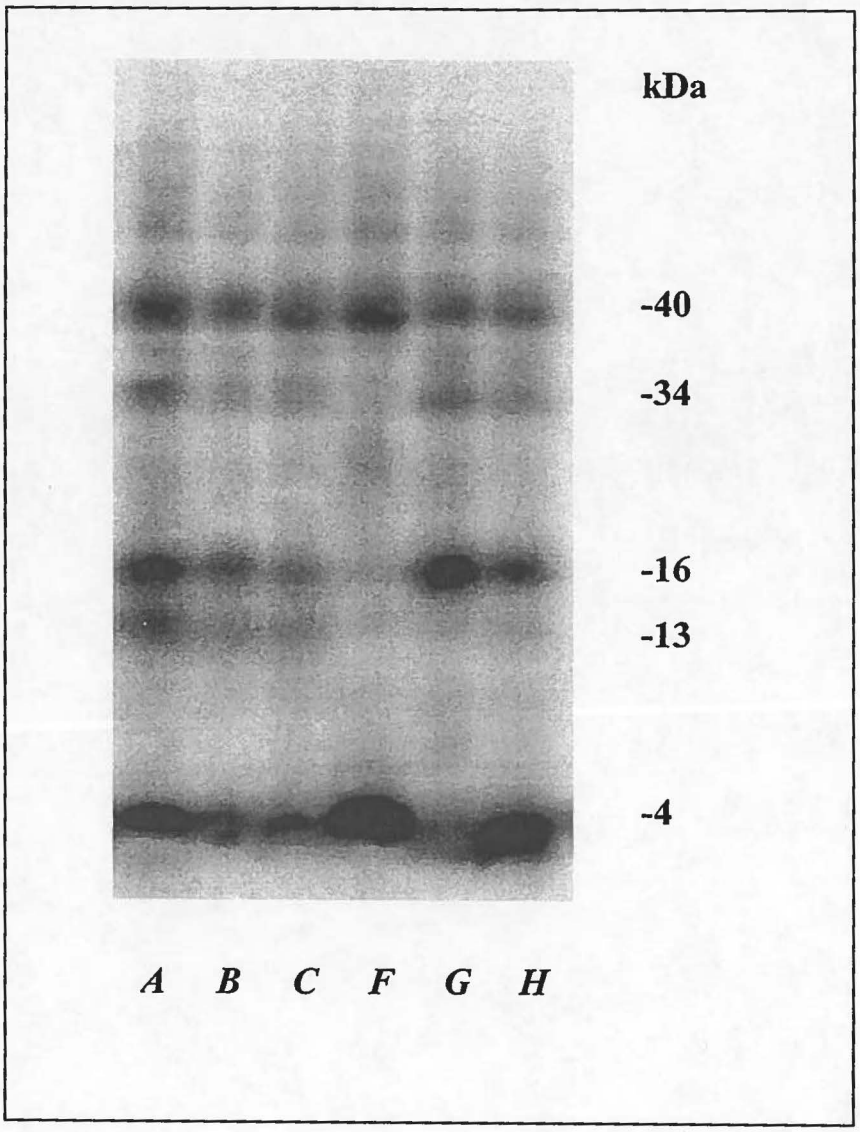


Figure 5

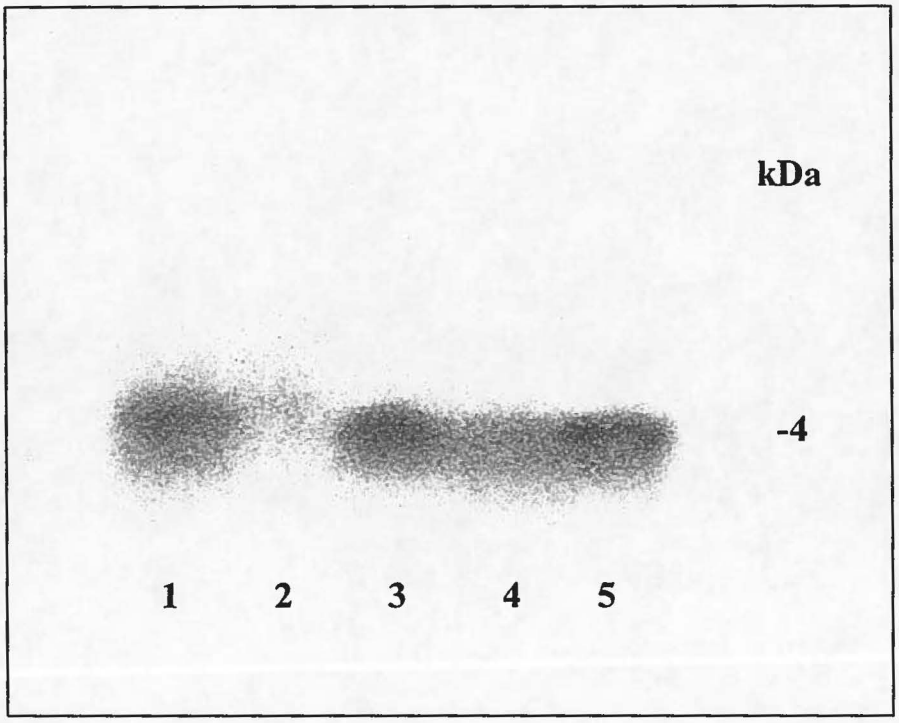


Table 1. pBNP1 and synthesized probes affinities toward NPR-A in bovine adrenal *zona glomerulosa* membrane preparations. Affinities are given as pK_i, -log K_d; mean ± standard error of two to four receptor binding assays with ^{125}I-rANP(1-28); ΔpK_i, pK_i of analog - pK_i of rANP(1-28). Non-specific binding (N) is given as a percentage of the coefficient given by the ratio of bound over free ligand assessed in saturating conditions.

Peptide	Acronym	subs.	Potency		^{125}I-Analog	
			Δ pK_i	rel. potency (%)	N (%)	Inc. (%)
[N, C, rANP(1-28)]pBNP32	pBNP1		0.53 ± 0.13	> 300	2	
[N^{α}-*p*-benzoylbenzoyl, Tyr^{2}]pBNP1	pBNP1-A	N-term.	0.11 ± 0.11	100	34	18
[N^{α}-*p*-benzoylbenzoyl, Tyr^{18}]pBNP1	pBNP1-B	N-term.	0.00 ± 0.10	100	22	41
[Tyr^{2}, Bpa^{3}]pBNP1	pBNP1-C	N-term.	0.08 ± 0.19	100	20	6
[Tyr^{6}, Bpa^{9}]pBNP1	pBNP1-D	Cycle	-1.76 ± 0.08	2		
[Bpa^{16}, Tyr^{18}]pBNP1	pBNP1-E	Cycle	-0.43 ± 0.10	36	15	0
[Bpa^{18}, Tyr^{26}]pBNP1	pBNP1-F	Cycle	0.14 ± 0.11	100	14	25
[Tyr^{18}, Bpa^{26}]pBNP1	pBNP1-G	C-term.	-0.04 ± 0.08	100	7	40
[Tyr^{18}, Bpa^{28}]pBNP1	pBNP1-H	C-term.	0.03 ± 0.11	100	6	40

Troisième Partie

3. Conclusion

Encore peu de publications concernent le site de reconnaissance du récepteur NPR-A. Pourtant, la nature homodimérique de ce récepteur, ainsi que l'homologie que ce dernier enregistre avec les récepteurs à activité tyrosine kinase, font de la dynamique de liaison une facette essentielle à la compréhension du mécanisme de transduction opéré par les peptides natriurétiques. Les récepteurs du PDGF (*platelet-derived growth factor*), et de GH (*growth hormone*), tous deux membres de la famille des protéines à activité tyrosine kinase, soulignent bien l'ingéniosité dont peut faire preuve la nature en ce domaine. Récepteurs homodimériques, ils interagissent avec leur ligand de façon différente. Le PDGF, lui-même dimérisé, se lie à deux sites identiques, un sur chacune des sous-unités protéiques, alors que GH, monomérique, procède à une liaison séquentielle faisant intervenir un premier site de liaison, ce qui entraîne la dimérisation du récepteur et rend accessible un second site de liaison, distinct du premier. Dans le cas du récepteur NPR-A, les données actuelles ne permettent pas de déterminer si le ligand interagit simultanément avec les deux sous-unités, et donc, si le ligand affiche un ou plusieurs épitopes. Ce mémoire rend compte de la démarche scientifique entreprise afin d'amener des éléments de réponse à cette question.

Si les travaux dont font l'objet ce mémoire n'apportent aucune réponse définitive quant au nombre ou à la nature des épitopes affichés par les peptides natriurétiques, ils auront néanmoins permis le développement d'outils puissants. Les travaux présentés ici confirment la pertinence de l'utilisation du pBNP1 comme gabarit de synthèse pour la production de sondes. Six nouveaux analogues photoactivables de très haute affinité sont maintenant disponibles. Ces analogues confirment également le choix des substitutions faites sur la base des études de structure-activité. Résultat très intéressant à souligner, la sonde pBNP1-F, ([Bpa^{16}, Tyr^{18}]pBNP1), qui incorpore le résidu benzophénone à l'intérieur du cycle peptidique, génère, une fois liée au récepteur, un profil de digestion au bromure de cyanogène distinct, et encore jamais publié. Le comportement de cet analogue en fait un candidat de choix pour de futures expérimentations, car il suggère la présence d'un second épitope.

À mon sens, ces travaux ont permis de jeter les bases pour le développement éventuel d'une sonde bisubstituée, qui, incorporant un benzophénone en position seize, et un second en position N ou C-terminal, permettra peut-être de 'capturer' simultanément deux sites de liaison du récepteur dimérique.