JOURNAL OF RECEPTOR RESEARCH

Volume 11, Numbers 1 - 4, 1991

JOURNAL OF RECEPTOR RESEARCH, 11(1-4), 259-273 (1991)

DISCRIMINATION AND QUANTIFICATION OF GLOMERULAR RECEPTOR SUBTYPES FOR ATRIAL NATRIURETIC FACTOR (ANF)

M. C. Kollenda, R. M. Scarborough, and A. L. Gerbes*
Dept. of Medicine II, Klinikum Großhadern, Marchioninistraße 15,
8000 München 70, University of Munich, W. Germany,
and Cal. Biotech., Mountain View, USA.

ABSTRACT

Binding sites for atrial natriuretic factor (ANF) were determined on isolated rat glomeruli as well as on glomerular membranes. To define optimal conditions, binding of ANF was investigated varying incubation time, temperature and protein concentration. Binding conditions were found to be best at 4°C for 5 hours with 15 μg of glomerular protein. Saturation and affinity cross-linking experiments confirmed the presence of two distinct receptor subtypes - the B-receptor (130 kDa) and the C-receptor (65 kDa). Quantitative differentiation of both ANF binding sites was achieved by competitive displacement with two different unlabeled ANF ligands: a) rANF(99-126) (homologous displacement), b) des(18-22)rANF(4-23)NH_2 (heterologous displacement). Intact glomeruli and glomerular membranes did not differ significantly in receptor density for the B-receptor (71 ± 37 vs. 94 ± 53 fmol/mg protein) or the C-receptor (976 ± 282 vs. 966 ± 167 fmol/mg protein) or in affinity constants for the B-receptor (43 ± 36 vs. 52 ± 44 pM) or the C-receptor (876 ± 377 vs. 307 ± 36 pM). Glomerular membranes compared to glomeruli showed less nonspecific binding and less intra-assay variation of measuring points done in triplicates.
This method of selective displacement should allow to study the influence of various physiological and pathophysiological conditions on the binding properties of B- and C-receptors for ANF.

INTRODUCTION

Atrial natriuretic factor (ANF), a peptide hormone, is released into the circulation by atrial cardiomyocytes (1). Its natriuretic, diuretic, vasorelaxant and hypotensive actions are mediated by interacting with two types of receptors. It has been reported that the B-receptor which is tightly coupled to guanylate cyclase exerts the biological effect (2) whereas the C-receptor presumably acts as a clearance receptor (3, 4) - its biological role, however, remains incompletely understood. Recent investigations demonstrated a negative coupling of C-receptors to adenylate cyclase (5) implying a more complex biological action than merely modulating ANF plasma concentrations. ANF receptors have been demonstrated on a variety of tissues (6, 7). A major target site for ANF in the kidney seems to be the glomerulus (8, 9). Binding studies have employed both - intact glomeruli and glomerular membranes, but binding properties of both tissues have not been compared so far. Furthermore, as yet only few studies have reported quantitative binding analysis of the two distinct ANF receptors (4, 10, 11). Therefore, aim of this study was to define optimal binding conditions to quantitatively differentiate B- and C-receptors.

MATERIALS AND METHODS

Isolation of Glomeruli and Preparation of Glomerular Membranes

Kidneys from male Sprague-Dawley rats (300-350 g) were used for tissue preparation. Glomeruli were isolated by a modified graded sieving method (12). Kidneys were cut longitudinally and the medulla was removed. All subsequent steps were performed in siliconized glassware at $4^{o}C$. The cortical tissue was pressed through a 250 μm stainless steel sieve. The fragments were collected and suspended in ice-cold phosphate-buffered saline (PBS), pH 7.5. This material was gently filtered through a 75 μm nylon sieve. Tissue remaining on this sieve was recovered and filtered through a 140 μm nylon sieve, allowing to separate glomeruli from

remaining tubules. Glomeruli thus retrieved were visualized under light microscopy and were devoid of tubules and vessels (purity ≥ 97%). Membrane preparation was then proceeded by centrifugation (10,000 g, 4°C, 10 min). Glomeruli were resuspended in 50 mM tris(hydroxymethyl)-aminomethane (Tris)•HCl, pH 7.4, 1mM $NaHCO_3$, and homogenized with a Polytron (Kinematica, Littau, Switzerland), at 4°C, setting 6 for 3 x 20s. The homogenate was diluted (1:1) with 50 mM Tris•HCl, pH 7.4, 1mM EDTA, 1mM $MgCl_2$ and sedimented at 30,000 g for 15 min at 4°C. The pellet was washed once and resuspended in 50 mM Tris•HCl, pH 7.4, 250 mM sucrose. The membranes were frozen and stored at -70°C. Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin as standard.

Binding Assay

To define optimal conditions, binding studies were performed in dependance of incubation time, temperature and protein concentration. Finally 15 μg of glomerular protein were incubated in triplicates with 20 pM ^{125}I-rANF (specific activity 2.200 Ci/mmol, Biotrend, Köln, W. Germany) at 4°C for 5 h in a final volume of 0.5 ml. The assay buffer contained: 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.5, 5 mM $MgCl_2$, 0.2% bovine serum albumin (BSA), 0.1% bacitracin and the following protease inhibitors: 4 μM aprotinin, 8 μg/ml phosphoramidon, 2 μg/ml bestatin, 40 μg/ml chymostatin (Sigma, Deisenhofen, W. Germany). For competitive binding studies (triplicates) the unlabeled peptides a) rANF(99-126) (Novabiochem, Läufelfingen, Switzerland) or b) des(18-22)rANF(4-23)NH_2, a truncated ANF analogue that binds to C-receptors only (4), were present in increasing concentrations (10^{-12}-10^{-6} M). Displacement with these two unlabeled ANF peptides as well as displacement of ^{125}I-rANF by unlabeled rANF(99-126) in presence of 10^{-6} M des(18-22)rANF(4-23)NH_2 allowed for differentiation of both receptor types. Saturation experiments contained 10-2.000 pM ^{125}I-rANF. Nonspecific binding was determined in presence of 10^{-6} M rANF(99-126). Reactions were stopped by adding 2.5 ml ice-cold 50 mM Tris•HCl, pH 7.4

and rapid filtration through 1% polyethyleneimine-treated Whatman GF/C filters. Filters were washed with 5 ml 50 mM Tris·HCl, pH 7.4, allowed to dry, and radioactivity was measured in an LKB 1261 Multigamma counter (Turku, Finland) with 80% efficiency.

Affinity Cross-linking of ANF Receptors

30 μg glomerular membrane protein were incubated for 5 h at 4°C with 60 pM ^{125}I-rANF(99-126) in the presence or absence of unlabeled rANF(99-126). After incubation, the membranes were washed, resuspended in ice-cold PBS-buffer, pH 7.5 and incubated with 0.1 mM bis(sulfosuccinimidyl)suberate in PBS or 0.2 mM disuccinimidyl suberate in dimethylsulfoxide (Pierce, Beijerland, Netherlands) for 25 min at 4°C. The reaction was quenched by adding 10 mM ammonium acetate and the membranes were centrifuged in an Eppendorf centrifuge for 7 min. The pellet was resuspended in 30 μl sample buffer (62 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue) containing 5% 2-mercaptoethanol and heated for 3 min at 100°C. The membranes were resolved on a 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel under constant voltage conditions (Hoefer Scientific Instruments, San Francisco, USA). Standard molecular weight markers were used for calculation of molecular weights of labeled bands (Serva, Heidelberg, W. Germany). Gels were stained, dried and exposed to Kodak X-OMAT AR film with a Kodak X-OMAT regular enhancing screen at -70°C for 14 days.

Analysis of Data

The results are expressed as mean ± SE for separate experiments. Saturation experiments were analyzed by ENZFITTER (Elsevier, BIOSOFT, Cambridge, United Kingdom). Data from competitive displacement curves were analyzed with the LIGAND program, based on a nonlinear least-squares curve-fitting (14). According to a model of two independent classes of binding sites, curves of homologous displacement (unlabeled ligand: rANF(99-126)) and heterologous displacement (unlabeled ligand:

des(18-22)rANF(4-23)NH_2) were analyzed simultaneously. This combined fitting of two different experiments allowed the estimation of receptor density of B- and C-receptors and of their equilibration dissociation constants.

RESULTS

Binding Assay: Characterization of Receptor Binding

Binding of ^{125}I-rANF to glomeruli and glomerular membranes was determined as a function of protein concentration, incubation time and temperature. As seen in Fig. 1 specific binding of ANF increased with increasing protein concentration, showing linearity between 5 μg and 30 μg for glomeruli as well as glomerular membranes. Incubation of glomeruli and glomerular membranes at 4^{o}C and 22^{o}C (Fig. 2) showed that binding of ^{125}I-rANF to glomerular membranes reached an equilibrium at both temperatures at 5 h which remained stable for a further 4 h. A quite similar binding curve was obtained with glomeruli. At 22^{o}C an equilibrium occured after 4 h, at 4^{o}C after 6 h. Nonspecific binding defined as the amount of ^{125}I-rANF bound in presence of 10^{-6} M unlabeled rANF(99-126) contributed always less than 4% (glomeruli) and less than 3% (glomerular membranes) of the total radioactivity added. In relation to the total binding however, nonspecific binding amounted up to 35% for glomeruli and up to 20% for glomerular membranes (Fig. 2). Higher specific binding (by about 10%) exhibited by glomerular membranes as compared to glomeruli could be observed at 22^{o}C as well as at 4^{o}C. Binding experiments at 4^{o}C however showed higher specific binding for glomeruli as well as for membranes by about 10% compared to binding curves done at 22^{o}C (Fig. 2). There was no significant degradation of ^{125}I-rANF during the incubation time of 5 h as determined by HPLC (data not shown). Based on these experiments further binding studies were performed at 4^{o}C for 5h with 15 μg of glomerular protein.

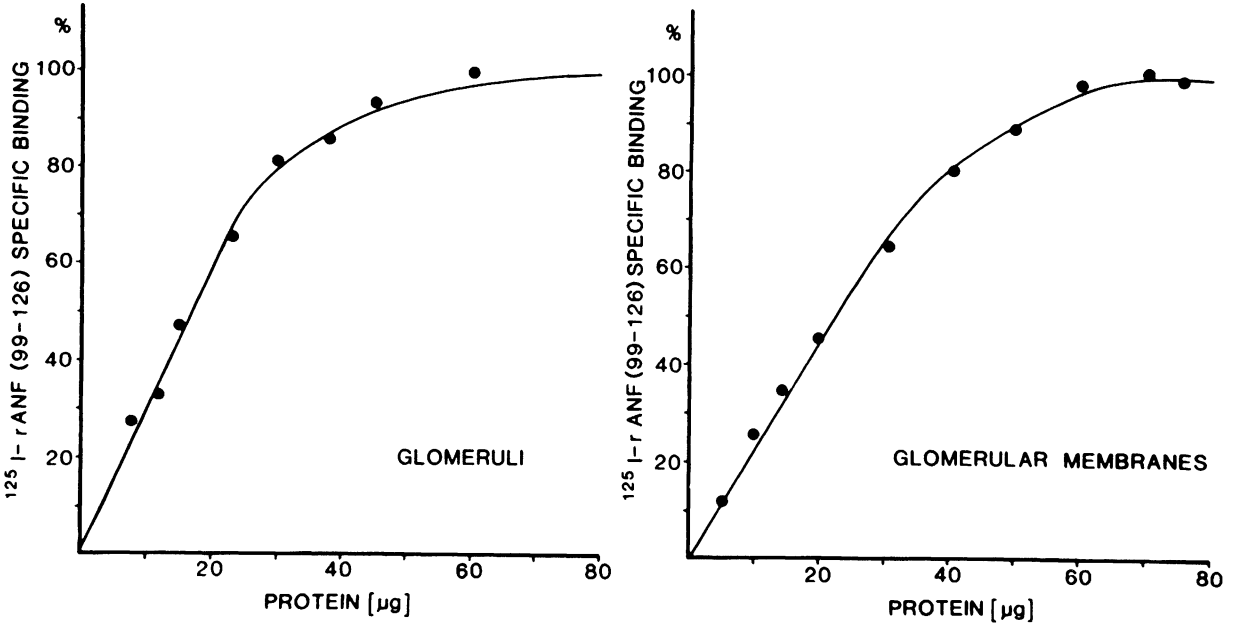

FIG. 1. Binding of ^{125}I-rANF(99-126) to rat glomeruli and glomerular membranes as a function of protein concentration. Tissue preparations were incubated at 4°C for 5 h with 20 pM ^{125}I-rANF.

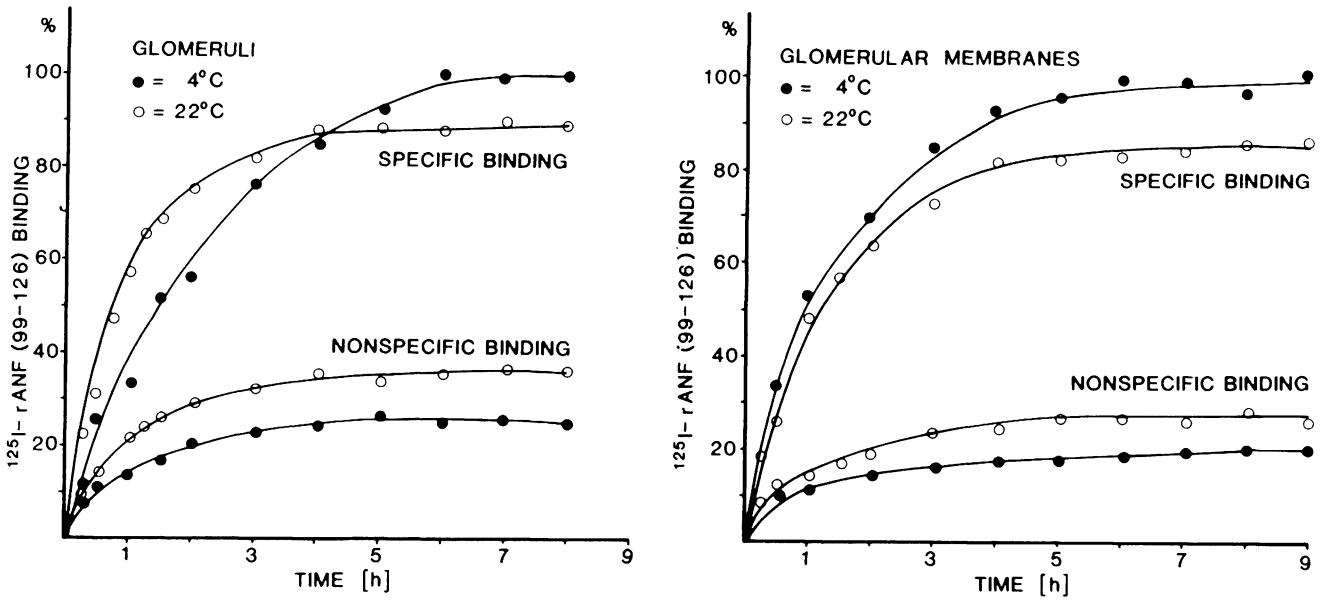

FIG. 2. Binding of ^{125}I-rANF(99-126) to rat glomeruli and glomerular membranes as a function of incubation time and temperature, respectively. The protein concentration was 15 μg for each tube.

Confirmation of two distinct ANF Receptor Subtypes

The binding of ^{125}I-rANF to glomerular membranes was saturable in a biphasic fashion. Scatchard analysis of data from saturation experiments demonstrated the presence of two distinct ANF binding sites with high and low affinity (data not shown).

Affinity cross-linking studies were employed to characterize the relative molecular weight (Mr) of both receptor types. The autoradiogramm of SDS-polyacrylamide gel electrophoresis-solubilized glomerular membranes showed two distinct bands with a Mr of 130 kDa and 65 kDa, respectively. The specifity of ^{125}I-rANF cross-linking was demonstrated by displacement with increasing concentrations of unlabeled rANF(99-126). The intensity of labeling of both receptors diminished in a dose-dependent manner and labeling was absent in presence of 10^{-6} M rANF (data not shown).

Confirmation of two receptor populations present on glomerular membranes was furthermore achieved by several types of displacement experiments. As shown in Fig. 3 competitive binding of labeled and unlabeled rANF(99-126) clearly demonstrated receptor heterogeneity. The displacement of bound ^{125}I-rANF by unlabeled des(18-22)rANF(4-23)NH_2 was used to estimate C-receptor density. In a further experimental approach competitive binding of labeled and unlabeled rANF(99-126) in presence of excess des(18-22)rANF(4-23)NH_2 allowed selective saturation of C -receptors and therefore verification of a second class of receptors (B-receptors) (Fig. 3).

Quantification of ANF Receptor Types

Data were obtained by displacement experiments from 10 animals. A representative displacement curve with both tissue preparations is demonstrated in Fig. 4. Table 1 shows the receptor density and affinity of B- and C-receptors calculated from glomeruli and glomerular membranes, respectively. There was no significant difference in receptor density or in affinity constants between glomeruli and glomerular membranes.

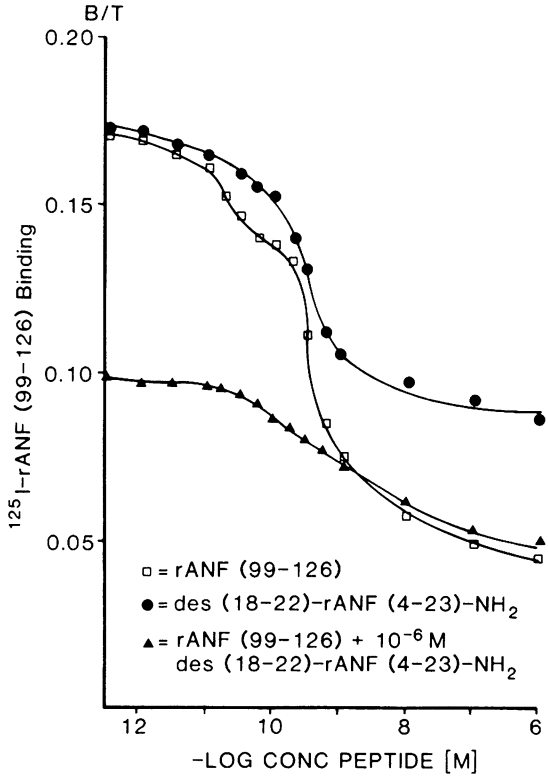

FIG. 3. Representative displacement binding curves of ^{125}I-rANF(99-126). 30 μg of glomerular membrane protein were incubated in presence of varying concentrations of unlabeled rANF(99-126) or des(18-22)rANF(4-23)NH_2 and of rANF(99-126) in presence of excess concentrations (10^{-6} M) of des(18-22)rANF(4-23)NH_2.

DISCUSSION

The purpose of the present study was to define optimal conditions for the quantitative differentiation of glomerular B- and C-receptors for ANF. In a first step binding of ^{125}I-rANF was investigated under various conditions. ANF binding to glomeruli and glomerular membranes as a function of time showed that equilibrium was reached between 4 and 5 h at 4^{o}C

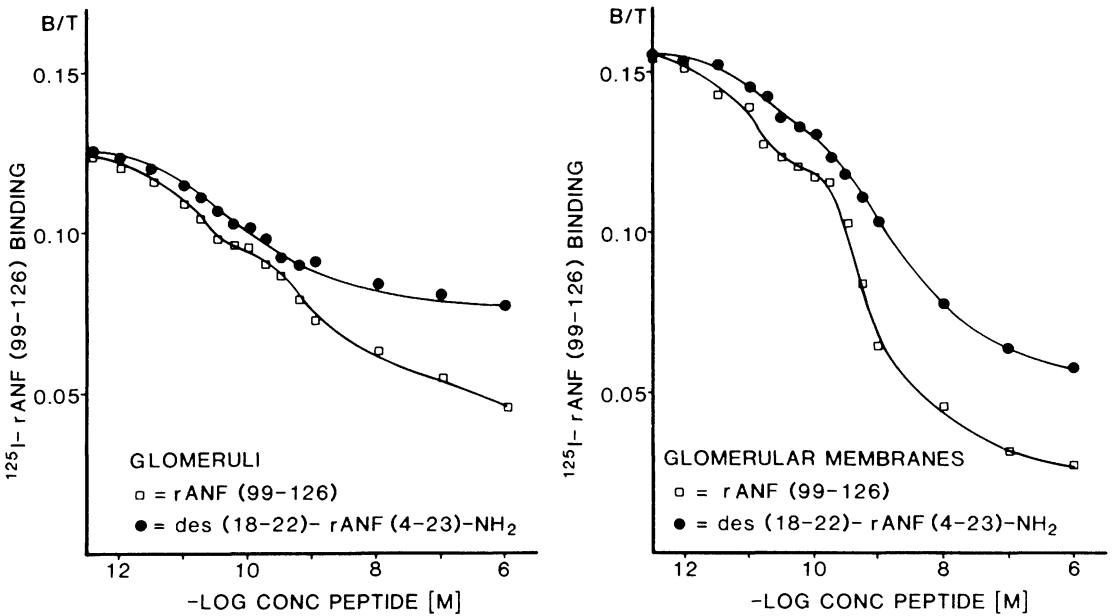

FIG. 4. Representative displacement binding curves of ^{125}I-rANF(99-126) to glomeruli and to glomerular membranes. Tissue preparations were incubated with varying concentrations of unlabeled rANF(99-126) or des(18-22)rANF(4-23)NH_2. Results are expressed as bound-total (B/T) vs. total (unlabeled ligand).

TABLE 1

Binding Properties of ANF Receptor Subtypes

	B-receptor		C-receptor	
	B_{max} [fmol/mg prot.]	K_D [pM]	B_{max} [fmol/mg prot.]	K_D [pM]
Glomeruli	71 ± 37	43 ± 36	976 ± 282	876 ± 377
Glom. Membr.	94 ± 53	52 ± 44	966 ± 167	307 ± 36

and 22°C. Studies on the time course of ANF binding to various renal tissues at 4°C and 22°C reported equilibration times between 60-90 min (15, 16) and 4 h (17, 18). The use of different assay buffers might account for these variations.

After the demonstration of a linear relationship of specific binding and protein concentration, optimal conditions for binding assays were defined as follows: 4°C (higher specific binding), 5 h incubation time (equilibrium), 15 μg protein (within the linear range). Under these conditions glomerular membranes compared to glomeruli showed higher specific binding and less intra-assay variation of the measuring points done in triplicates. This finding may be due to a more homologous tissue preparation of glomerular membranes.

In a second step the presence of two distinct ANF receptors on glomerular membranes was confirmed by saturation binding and by affinity cross-linking studies. Scatchard analysis of data from saturation experiments was most consistent with a model for two separate classes of receptors, differing in their affinity and binding capacity. The autoradiogramm of SDS polyacrylamide gel electrophoresis-resolved glomerular membranes revealed two labeled bands with molecular weights of 130 kDa and 65 kDa, as reported for the B- and C-receptor, respectively

(10, 19, 20, 21). These two experiments most likely identified the two receptors as B- and C-receptors.

In a third step we investigated a technique that would allow to quantitatively distinguish B- and C-receptors. It has been reported that B- and C-receptors differ in their structural requirements for binding of ANF ligands (22, 23, 24). While B-receptors bind full-length disulfide-bridged ANF ligands only, C-receptors exhibit less stringent requirements and are able to bind truncated ANF analogues as well. Utilizing this property we chose an experimental approach of homologous (unlabeled ligand: rANF(99-126)) and heterologous displacement (unlabeled ligand: des(18-22)rANF(4-23)NH_2, binding to C-receptors only). Data analysis with the LIGAND program then allowed for a quantitative discrimination of both ANF receptor types. In both tissue preparations, glomeruli as well as glomerular membranes, the B-receptor constituted 7-9% of the total ANF binding sites. Other investigators reported ratios for B-receptors over the total number of receptors on glomeruli between 1 and 40% (4, 10). These variations probably depend on different ways of tissue preparation, purification and data analyzing techniques. Binding affinity constants seem to be less influenced by experimental maneuvers. Previous reports of K_D values for B- and C-receptors of glomeruli and glomerular membranes are in agreement with our results (4, 10).

In conclusion, we have demonstrated that ANF binds to two separate types of receptors on glomerular tissue. We have determined appropriate conditions for selective displacement allowing to quantitatively differentiate B- and C-receptors and to determine their binding affinity constants. Since glomerular membranes exhibit higher specific binding and less intra-assay variation than glomeruli we favor using glomerular membranes for quantitative ANF binding studies.

This method of competitive displacement with two different ligands should allow to investigate possible influences of various physiological and pathophysiological conditions on the binding properties of B- and C-receptors for ANF (25).

ACKNOWLEDGMENTS

We thank Dr. A. M. Vollmar for helpful discussions, Dr. P. J. Munson and Prof. J. Remien for clarification of data analysis and Prof. G.

Paumgartner for continuous support. This work was supported by a grant from the Deutsche Gesellschaft für Verdauungs- und Stoffwechselkrankheiten (Asche Stipendium of A. L. G.) and from the Deutsche Forschungsgemeinschaft (DFG Ge 576).

REFERENCES

1. De Bold A. J. Atrial natriuretic factor: a hormone produced by the heart. Science 230, 767-770, 1985.

2. Almeida, F. A.; Suzuki, M.; Scarborough, R. M.; Lewicki, J. A.; and Maack, T. Clearance function of type C receptors of atrial natriuretic factor in rats. Am. J. Physiol. 256, R469-R475, 1989.

3. Chinkers, M.; Garbers, D. L.; Chang, M.-S.; Lowe, D. G.; Chin, H.; Goeddel, D. V.; and Schulz, S. A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. Nature 338, 78-83, 1989.

4. Maack, T., Suzuki, M; Almeida, F. A.; Nussenzveig, D.; Scarborough, R. M.; McEnroe, G. A.; and Lewicki, J. A. Physiological role of silent receptors of atrial natriuretic factor. Science 238, 675-678, 1987.

5. Anand-Srivastava, M. B.; Sairam, M. R.; and Cantin, M. Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. J. Biol. Chem. 265, 8566-8572, 1990.

6. Mantyh, C. R.; Kruger, L.; Brecha, N. C.; and Mantyh, P. W. Localization of specific binding sites for atrial natriuretic factor in peripheral tissues of the guinea pig, rat and human. Hypertension 8, 712-721, 1986.

7. Stewart, R. E.; Swithers, S. E.; Plunkett, L. M.; and McCarthy, R. ANF receptors; distribution and regulation in central and peripheral tissues. Neurosci. Biobehav. Rev. 12, 151-168, 1988.

8. Bianchi, C.; Gutkowska, J.; Thibault, G.; Garcia, R.; Genest, J.; and Cantin, M. Radioautographic localization of ^{125}I-atrial natriuretic factor (ANF) in rat tissues. Histochemistry 82, 441-452, 1985.

9. Kim, J. K.; Summer, S. N.; Durr, J.; and Schrier, R. W. Enzymatic and binding effects of atrial natriuretic factor in glomeruli and nephrons. Kidney Int. 35, 799-805, 1989.

10. Féthière, J.; Meloche, S.; Nguyen, T. T.; Ong, H.; and De Léan, A. Distinct properties of atrial natriuretic factor receptor subpopulations in epithel and fibroblast cell lines. Mol. Pharmacol. 35, 584-592, 1989.

11. Martin, E. R.; Lewicki, J. A.; Scarborough, R. M.; and Ballermann, B. J. Expression and regulation of ANF receptor subtypes in rat renal glomeruli and papillae. Am. J. Physiol. 257, F649-F657, 1989.

12. Misra, R. P. Isolation of glomeruli from mammalian kidneys by graded sieving. Am. J. Clin. Pathol. 58, 135-139, 1974.

13. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275, 1951.

14. Munson, P. J.; and Rodbard, D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107, 220-239, 1980.

15. Carrier, F.; Thibault, G.; Schiffrin, E. L.; Garcia, R.; Gutkowska, J.; Cantin, M.; and Genest, J. Partial characterization and solubilization of receptors for atrial natriuretic factor in rat glomeruli. Biochem. Biophys. Res. Commun. 132, 666-673, 1985.

16. Gunning, M. E.; Ballermann, B. J.; Silva, P.; Brenner, B. M.; and Zeidel, M. L. Characterization of ANP receptors in rabbit inner medullary collecting duct cells. Am. J. Physiol. 255, F324-F330, 1988.

17. Hori, R.; Inui, K.; Saito, H.; Matsukawa, Y.; Okumura, K.; Nakao, K.; Morii, N.; and Imura, H. Specific receptors for atrial natriuretic polypeptide on basolateral membranes isolated from rat renal cortex. Biochem. Biophys. Res. Commun. 129, 773-779, 1985.

18. Ballermann, B. J. A highly sensitive radioreceptor assay for atrial natriuretic peptide in rat plasma. Am. J. Physiol. 254, F159-F163, 1988.

19. Cole, F. E.; Hamada, M.; Burmester, H. A.; Graci, K. A.; MacPhee, A. A.; Trippodo, N. C.; and Frohlich, E. D. Atrial natriuretic peptide binding properties of rat glomerular membranes.- in: Biologically active atrial peptides, edited by B. M. Brenner and J. H. Laragh, p 235-239, Raven Press, New York, N.Y., 1987.

20. Fuller, F.; Porter, J. G.; Arfsten, A. E.; Miller, J.; Schilling, J. W.; Scarborough, R. M.; Lewicki, J. A.; and Schenk, D. B. Atrial natriuretic peptide clearance receptor. J. Biol. Chem. 263, 9395-9401, 1988.

21. Leitman, D. C.; Andresen, J. W.; Catalano, R. M.; Waldman, S. A.; Tuan, J. J.; and Murad, F. Atrial natriuretic peptide binding, cross-linking, and stimulation of cyclic GMP accumulation and particulate guanylate cyclase activity in cultured cells. J. Biol. Chem. 263, 3720-3728, 1988.

22. Scarborough, R. M.; Schenk, D. B.; McEnroe, G. A.; Arfsten, A.; Kang, L.-L.; Schwartz, K.; and Lewicki, J. A. Truncated atrial natriuretic peptide analogs. J. Biol. Chem. 261, 12960-12964, 1986.

23. Porter, J. G.; Wang, Y.; Schwartz, K.; Arfsten, A.; Loffredo, A.; Spratt, K.; Schenk, D. B.; Fuller, F.; Scarborough, R. M.; and Lewicki, J. A. Characterization of the atrial natriuretic peptide clearance receptor using a vaccinia virus expression vector. J. Biol. Chem. 263, 18827-18833, 1988.

24. Bovy, P. R.; O'Neal, J. M., Olins, G. M.; and Patton, D. R. Identification of structural requirements for analogues of atrial natriuretic peptide (ANP) to discriminate between ANP receptor subtypes. J. Med. Chem. 32, 869-874, 1989.

25. Kollenda, M.C. "Veränderungen glomerulärer ANF Rezeptoren bei Leberzirrhose". Dissertation der Medizinischen Fakultät der Ludwig-Maximilians-Universität München, in preparation.