Specific membrane receptors for atrial natriuretic factor in renal and vascular tissues

(aorta/kidney cortex/vasorelaxant/diuretic/peptides)

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ABSTRACT Membranes from rabbit aorta and from rabbit and rat kidney cortex possess high-affinity ($K_d = 10^{-10}$ M) specific binding sites for atrial natriuretic factor (ANF). Similar high-affinity sites are present in an established cell line from pig kidney, LLC-PK1. Results of fractionation studies indicate that the receptors are localized in the plasma membrane of these tissues. The binding is time-dependent and saturable. An excellent quantitative correlation was found between the affinity of synthetic ANF and analogs of intermediate activity to aorta membranes and the half-maximal concentration needed for relaxation of rabbit aorta rings contracted by addition of serotonin. Furthermore, the binding affinity of the receptor in kidney membranes is consistent with the concentration required for in vivo natriuresis in the rat. Biologically inactive synthetic ANF fragments and other peptide hormones such as angiotensin II and vasopressin do not significantly inhibit binding. These data suggest that the receptors for ANF in vascular and renal tissues are responsible for mediating the physiological actions of this peptide in these target tissues.

A possible role for the cardiac atria in the regulation of extracellular fluid volume and electrolyte concentration has been shown by the induction of sodium and water excretion in response to changes in inatraial pressure and stretch of the atrial wall (1). The cardiac atria possess granules that have the appearance of secretory granules (2-4) and whose number can be altered by manipulation of the water-electrolyte balance in experimental animals (5). Crude extracts of atria from several species have been shown to possess potent diuretic and natriuretic activity when given intravenously to rats (6). Subcellular fractionation (7) and immunocytochemistry (8) indicated that these granules are storage sites for an atrial natriuretic factor (ANF). Furthermore, in vitro, atrial extracts were shown to be potent vasorelaxants (9-14). As little as 0.0006 atrial equivalent per milliliter gave 50% relaxation of precontracted aorta tissues (12). In vivo, ANF caused lowering of mean arterial blood pressure in normal and hypertensive animals (6, 15, 16).

Recently, a family of peptides has been isolated from acidic extracts of rat (10, 11, 19-22) and human (23) atria and sequenced independently by several laboratories. From rat atria, the longest peptide that has been reported contains 33 amino acids, 1, and was isolated as the free COOH-terminal tyrosine acid (20, 21). Shorter rat ANF peptides are truncated at either the NH$_2$ or COOH terminus.

H-Leu-Ala-Gly-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser
15
Cys-Phe-Gly-Gly-Arg-Ile-Asp-Ile-Gly-Ala
20
Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH
30

A peptide consisting of residues 8-33, designated sANF, has been synthesized (21, 24) and shown to possess full biological activity (12, 16, 21, 24). The IC$_{50}$ of sANF for relaxation of precontracted rabbit aorta rings is 550 pM (12). The amount of sANF required for half-maximal natriuresis when given intravenously by infusion to conscious rats is 60-100 pmol/kg-min and 3.6 pmol/kg-min when given directly into the renal artery of dogs (16). The biological activity and isolation of ANF were recently reviewed (25).

Although a physiological role for endogenous ANF is implied by the potent biological activity of these peptides, the existence of a circulating form of ANF or specific receptors for ANF in target tissues has not been demonstrated. In this report we describe specific receptors for ANF as defined by binding of radioiodinated sANF of high specific activity to membranes from vascular and renal tissues. The nonradioactive moniodotyrosine derivative of sANF (I-sANF) is shown to possess both vasorelaxant and natriuretic activity. A high-affinity receptor is present in aorta and kidney cortex and in an established kidney cell line, LLC-PK1 (26). The specificity of the $^{125}$I-sANF binding is demonstrated by markedly reduced binding in the presence of unlabeled sANF and analogs with similar biological profiles.

MATERIALS AND METHODS

Materials. The synthetic ANF peptides (1 and Table 2) were synthesized as described (21, 24). The following peptides were obtained as indicated: bovine pancreas insulin and bactracin, Sigma; angiotensin II and somatostatin-28, Peninsula Laboratories (San Carlos, CA); somatostatin-14, vasopressin, thyrotropin-releasing hormone, and vasotocin, Bachem (Torrance, CA); bovine serum albumin (BSA) (fraction V), Miles; substance P, prepared by W. J. Paleveda (Merck Sharp & Dohme Research Laboratories). Other reagents and solvents were of the highest purity available commercially. Glass fiber filters (GF/C) were obtained from Whatman and $\mu$Bondapac C$_{18}$ reversed-phase HPLC columns were from Waters Associates.

Methods. Iodination of sANF and HPLC purification. The monof$^{125}$Iiodotyrosine derivative of sANF was prepared by a modification of the method of Hunter and Greenwood (27) by using chloramine-T. Synthetic ANF, 10 $\mu$g in 10 $\mu$l of distilled H$_2$O, was combined with 50 $\mu$l of 0.5 M sodium phosphate (pH 7.6) and 1 mCi (1 Ci = 37 GBq) of Na$^{125}$I (Amersham). Iodination was initiated by addition of 10 $\mu$l of chloramine-T solution (1 mg/ml in 0.05 M sodium phosphate, pH 7.6). After 15 sec, the reaction was quenched by addition of 10 $\mu$l of sodium thiosulfate (2 mg/ml in 0.05 M sodium phosphate buffer) and the reaction mixture was immediately injected onto the C$_{18}$ reversed-phase HPLC column equilibrated in 0.01 M ammonium acetate (pH 4.0). Elu-

Abbreviations: ANF, endogenous atrial natriuretic factor; sANF, synthetic ANF-(8-33)-OH; I-sANF, moniodotyrosine derivative of sANF; BSA, bovine serum albumin.
Vasorelaxant activity in rabbit aorta rings. The vasorelaxant activity of sANF and synthetic fragments was determined as described (12). Thoracic aorta ring segments from New Zealand White rabbits (2.0–2.6 kg) were contracted by addition of serotonin (300 nM) with the vasodilator peptides added cumulatively once the contraction reached an equilibrated plateau. IC₅₀ values were obtained by linear regression analysis using logarithmic transformation of the concentrations.

RESULTS

Iodination and Characterization of ¹²⁵I-sANF. Iodination and HPLC purification of sANF with the present procedure results in a monooxidated peptide with maximal theoretical specific radioactivity of 600 μCi/μg, corresponding to one iodine per peptide molecule. The radioiodinated sANF was eluted from the reversed-phase HPLC column well separated (ca. 2 min) from the unlabeled and diiodo forms of sANF (Fig. 1). By using a low ratio of iodide to peptide during the radiiodination procedure, only a single iodinated product was obtained. Rechromatography of the ¹²⁵I-sANF sample showed that it was essentially homogeneous and was stable to storage at 4°C for several weeks. The antigenicity of the ¹²⁵I-sANF was retained as shown by immunoprecipitation with rabbit anti-ANF antibodies and by binding to an anti-ANF IgG affinity column. Enzymatic treatment of the ¹²⁵I-sANF sample with carboxypeptidase A, previously shown to remove only the COOH-terminal tyrosine from sANF (20), and subsequent HPLC chromatography of the reaction products resulted in the removal of >90% of the ¹²⁵I from the original peptide. Cochromatography with unlabeled monoo- and diiodotyrosine indicated that the radioiodinated product was mono[¹²⁵I]iodotyrosine (not shown). The diiodotyrosine derivative was formed as a minor product under conditions of a high iodide-to-tyrosine ratio.

Nonradioactive I-sANF was produced by a procedure similar to that described above (Fig. 1) in quantities sufficient for determination of biological activity. Under optimal conditions, conversion of sANF to I-sANF was ~48% based on


Fig. 1. HPLC separation of I-sANF (Upper) and ¹²⁵I-sANF (Lower) from a chloramine-T iodination reaction. Chromatography was on a μBondapak C₁₈ column, 0.39 × 30 cm, at ambient temperature, elution with a linear gradient of 0–50% acetonitrile in aqueous 0.05 M ammonium acetate (pH 4) and flow rate of 1 ml/min. One-minute fractions were collected. (Upper) Ultraviolet spectra were measured for collected fractions 24–30. Fractions containing sANF and I-sANF were pooled separately and lyophilized, and peptide content was determined by amino acid analysis. (Lower) Aliquots, 10 μl, were removed into vials for counting ¹²⁵I radioactivity. The tube with the highest ¹²⁵I activity was used for membrane binding studies.
on the absorbance of the respective HPLC fractions and by amino acid analysis. Iodinated sANF gave complete relaxation of serotonin-contracted rabbit (IC\textsubscript{50} = 1650 pM) and rat (IC\textsubscript{50} = 1050 pM) aortae but required a slightly higher concentration than sANF (550 pM and 850 pM, respectively) for 50% relaxation. In anesthetized rats, I-sANF and sANF produced equal natriuretic activity (5315 and 5998 μEq/min·μg, respectively). Previous studies have shown that the COOH-terminal tyrosine is not necessary for biological activity (10).

**Binding of \textsuperscript{125}I-sANF.** \textsuperscript{125}I-sANF was found to bind specifically to crude rabbit aorta membranes. Scatchard analysis of the data obtained in one experiment by competition with unlabeled sANF revealed a single high-affinity receptor species (K\textsubscript{d} = 1.29 × 10\textsuperscript{-10} M, 96 fmol/mg of protein, Fig. 2). Four replicate determinations resulted in a mean K\textsubscript{d} of 9.2 ± 2.3 × 10\textsuperscript{-11} M (mean ± SD) (see Table 2). Binding was linear over the range of 6–100 μg of membrane protein. By HPLC analysis, the bound ligand was identical to the original \textsuperscript{125}I-sANF sample.

The binding profile for \textsuperscript{125}I-sANF in crude membranes prepared from kidney cortex of rabbits was more complex than that observed for the rabbit aorta. The data were best described by a two-site model—e.g., a high- and a low-affinity site (Fig. 3). The low-affinity binding sites appeared in varying amounts in different preparations. The high-affinity sites have similar properties to the high-affinity sites in aorta membranes (K\textsubscript{d} = 5.2 × 10\textsuperscript{-11} M, 35 fmol/mg). Similar results were found for membranes prepared from either fresh or frozen tissues and for membranes stored at −80°C. Discontinuous sucrose gradient fractionation of these membranes (Table I) resulted in the 3-fold enrichment of the high-affinity sites and loss of the low-affinity sites in the low-density sucrose fraction that contains principally plasma membranes.

The specificity of sANF binding to the high-affinity rabbit aorta and renal receptors is shown by the absence of competition by several biologically inactive sANF intermediates (Table 2) and the unrelated peptide hormones, including angiotensin II, insulin, somatostatin-28, substance P, thyrotropin-releasing hormone, and vasopressin, at concentrations of 1 μM. Furthermore, the IC\textsubscript{50} values for vasorelaxation of sANF analogs parallel the K\textsubscript{d} values of these sANF analogs for binding to rabbit aorta membranes with reduced binding affinity observed for compounds having reduced vasorelaxant activity (Table 2). The consistency of the K\textsubscript{d} and IC\textsubscript{50} values (correlation coefficient = 0.86) for sANF in rabbit aorta provides evidence that this tissue is a primary target for the biological action of ANF.

In contrast to the results obtained in rabbit kidney membranes, only low-affinity binding was observed in crude membranes prepared by an identical procedure from rat kidney cortex. The crude rat kidney preparation was not stable to freezing and had markedly reduced binding activity when stored at 4°C for 24–48 hr. HPLC analysis of the free and membrane-bound ligand revealed extensive degradation of the ligand during the 30-min incubation at 0°C. Partial purification of the crude membranes by discontinuous sucrose gradient centrifugation removed most of the degrading activity and revealed high-affinity sites (K\textsubscript{d} = 4.9 × 10\textsuperscript{-11} M) in the low-density plasma membrane fraction.

Membranes have also been prepared from the homogenous population of LLC-PK\textsubscript{1}, kidney cells by procedures described above. Preliminary experiments have revealed a single class of high-affinity sites (K\textsubscript{d} = 1.98 × 10\textsuperscript{-10} M) and a similar density of sites (65 fmol/mg of protein) as observed in the rabbit and rat renal membranes.

**DISCUSSION**

Conditions have been optimized for the preparation of an iodinated analog of sANF with retention of both vasorelaxant and natriuretic activity. Radiolabeled moniodinated sANF was prepared by a similar procedure and, when separated from unlabeled sANF and \textsuperscript{125}I-sANF by reversed-phase HPLC, has maximal specific radioactivity and good long-term stability. High specific binding of \textsuperscript{125}I-sANF was

**Table 1. Discontinuous sucrose gradient fractionation of rabbit kidney membranes**

<table>
<thead>
<tr>
<th>Membrane fraction</th>
<th>Specific \textsuperscript{125}I-sANF bound, fmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude membrane</td>
<td>6.2</td>
</tr>
<tr>
<td>Gradient fraction</td>
<td></td>
</tr>
<tr>
<td>1 (top)</td>
<td>15.9</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Binding of \textsuperscript{125}I-sANF was carried out at 4°C for 1 hr in 50 mM Hepes, pH 7.5/5 mM MgCl\textsubscript{2}/0.2% BSA/0.1% bacitracin in a total volume of 0.5 ml. Each tube contained 100 μg of protein and 24 pm of \textsuperscript{125}I-sANF. Specific binding is defined as the difference between total counts bound in the absence and presence of 10\textsuperscript{-6} M unlabeled sANF.
detected in membranes prepared from vascular and renal tissues and from an established kidney cell line. Low nonspecific binding (<2% of the total radioactivity added) was observed to the polyethyleneimine-treated glass fiber filters or to the membrane preparations in the presence of 1 μM sANF.

High-affinity sANF receptor sites are present in membranes derived from rabbit aorta. The specificity of binding of [125I]-sANF is shown by inhibition of binding with biologically active sANF intermediates and not by inactive intermediates or by nonrelated peptide hormones. The observed dissociation constant is consistent with the concentration of sANF or its analogs needed for relaxation of rabbit aortic rings.

Similar high-affinity receptors were found in renal tissues. In some preparations of rabbit kidney membranes, high- and low-affinity sites were detected. However, partial purification of the membranes by sucrose gradient centrifugation resulted in an enrichment of the high-affinity binding sites in the plasma membrane fraction with removal of the apparent low-affinity binding. The rat kidney cortex membranes also possess the high-affinity sites that were only observed after partial purification of the crude membranes. HPLC analysis of the [125I]-sANF from the binding mixture indicated varying degrees of degradation of the ligand in the crude rat and rabbit preparations. This observation probably accounts for the apparent low-affinity sites.

The LLC-PK₁ cells are a homogeneous population established from porcine kidney that retain characteristics of polar transporting epithelial cells. The high-affinity sites observed in the rat and rabbit kidney membranes could be of vascular origin since the kidney cortex contains many cell types, including vascular smooth muscle. However, the presence of similar high-affinity sites on the LLC-PK₁ cells indicates that the kidney receptor is probably of renal tubular origin.

The site of action of ANF in the kidney has not yet been defined. Micropuncture studies indicate that ANF exerts its effects in the distal tubules (32, 33). ANF does not alter the active sodium ion transport by inhibition of the Na⁺,K⁺-ATPase (34-36) or sodium ion flux in the proximal tubule as measured by Na NMR and tubular oxygen consumption (M. J. Avison, S. Gullans, and R. Shulman, personal communication) or oxygen consumption in kidney slices (34).

The LLC-PK₁ cells exhibit properties that are consistent with both distal (37-39), including the medullary portion of the thick ascending limb of the loop of Henle (39), and proximal tubules (26, 40, 41). The demonstration of specific high-affinity ANF receptors in these cells makes this cell line a possible model system for studying the direct effect of ANF on renal cells.

The relevance of ANF receptors to the biological action of ANF is implied by the correlation of the binding constants with the IC₅₀ values for vasorelaxation and the Kₐ values for receptor binding in aorta was 0.86.

Table 2. Vasorelaxant (IC₅₀) and receptor binding (Kₐ) activities of ANF peptides

<table>
<thead>
<tr>
<th>ANF analog</th>
<th>Rabbit aorta¹</th>
<th>Rabbit kidney§</th>
</tr>
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<tbody>
<tr>
<td>H-(8-33)-OH</td>
<td>5.5 × 10⁻¹⁰</td>
<td>0.9 × 10⁻¹⁰ (4)</td>
</tr>
<tr>
<td>H-(8-33)-NH₂</td>
<td>5.0 × 10⁻¹⁰</td>
<td>0.7 × 10⁻¹⁰ (1)</td>
</tr>
<tr>
<td>Boc-(8-33)-OH</td>
<td>4.0 × 10⁻¹⁰</td>
<td>7.2 × 10⁻¹⁰ (1)</td>
</tr>
<tr>
<td>Boc-(8-33)-NH₂</td>
<td>4.0 × 10⁻¹⁰</td>
<td>3.0 × 10⁻¹⁰ (1)</td>
</tr>
<tr>
<td>Boc-<a href="8-33">AcetylCys¹²,²⁰</a>-OH</td>
<td>4.0 × 10⁻⁸</td>
<td>4.4 × 10⁻⁸ (1)</td>
</tr>
<tr>
<td>Boc-<a href="8-33">AcetylCys¹²,²⁰</a>-NH₂</td>
<td>0.9 × 10⁻⁸</td>
<td>1.0 × 10⁻⁸ (1)</td>
</tr>
<tr>
<td>Boc-<a href="8-15">AcetylCys¹⁰</a>-OCH₃</td>
<td>&gt;10⁻⁷</td>
<td>&gt;10⁻⁷ (1)</td>
</tr>
<tr>
<td>H-<a href="22-33">AcetylCys¹⁰</a>-OH</td>
<td>&gt;10⁻⁷</td>
<td>&gt;10⁻⁷ (1)</td>
</tr>
</tbody>
</table>

Boc, N-butoxy carbonyl; Ac, acetylamidomethyl.

¹Variation of the Kₐ values (n values in parentheses) for replicate determinations (n > 1) was 25–60%.
²The correlation coefficient between the IC₅₀ values for vasorelaxation and the Kₐ values for receptor binding in aorta was 0.86.
³Serotonin contracted, 300 nM; 95% confidence limits are 18–47% for four or more replicates.
⁴Crude membrane fraction.
§Partially purified plasma membrane fraction.

Note Added in Proof. The presence of high-affinity specific receptors for ANF which mediate the inhibitory action of ANF in stereototyping has also been observed in bovine adrenal glomerulosa cells [observed by De Leen et al. (42, 43)].

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