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STIMULATION OF GUANYLATE CYCLASE ACTIVITY BY IRREVERSIBLE BINDING OF ATRIAL NATRIURETIC PEPTIDE TO ITS RECEPTOR

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Abstract—We examined receptor binding profiles of atrial natriuretic peptide (ANP) in rat tissue using ^{125}I-labeled α-rat ANP ([^{125}I]α-rANP). Specific [^{125}I]α-rANP binding to its receptor was reversible following addition of unlabeled free α-rANP, but it became increasingly irreversible with time during incubation. Irreversible binding of α-rANP was observed both at 0° and 25° in homogenates of adrenal capsules and lungs, crude membranes of renal glomeruli, partially purified membranes of lung, solubilized membrane preparations from renal glomeruli, and intact renal glomeruli. Irreversible binding increased in a time- and temperature-dependent manner. HPLC analysis demonstrated that the irreversibly bound radioactivity, which was extracted by 1 N CH_3COOH from both intact renal glomeruli and from partially purified membranes, was associated with intact [^{125}I]α-rANP. Irreversibly bound α-rANP increased cGMP concentrations by activating guanylate cyclase activity. These findings suggest that the appearance of irreversible binding of α-rANP to its receptor is independent of its internalization, and may be involved in message transduction and subsequent biological responses.

Atrial natriuretic peptide (ANP) is a hormone which regulates water and electrolyte homeostasis. The hormone expresses its actions through specific receptors present on plasma membranes of target tissues. Autoradiographic studies of [^{125}I]α-rANP† (3–28) injected into rats revealed the presence of widespread binding sites for this peptide hormone [1]. Kidney, adrenal gland, lung and small intestine are the major organs which have dense binding sites for the hormone. Renal glomerulus has the highest density of specific binding sites [1]. The hormone has a diuretic/natriuretic action in the kidney [2], is a vasodilator [3, 4], and inhibits aldosterone secretion from the adrenal cortex [5]. It also reduces edema in the lung [6] and stimulates Na^+-dependent water uptake in the small intestine [7].

The initial event triggered by ANP is an elevation of cGMP levels in target tissues [8, 9], which is due to the activation of particulate guanylate cyclase [10, 11]. This fact suggests that the ANP receptor may be coupled to guanylate cyclase. We have been interested in this coupling process and have performed experiments in order to clarify how the signal from the occupied ANP receptor is transduced to the cyclase. In this paper we report that there are apparently two modes of binding of α-rANP to its receptor, i.e. reversible and irreversible, and that the two modes of binding represent two distinguishable states of a single α-rANP–receptor complex. The irreversibly bound α-rANP–receptor complex is the major species that induces the activation of guanylate cyclase.

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† Abbreviations: α-rANP, α-rat atrial natriuretic peptide; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TFA, trifluoroacetic acid; and IBMX, 3-isobutyl-1-methyl xanthine.

MATERIALS AND METHODS

Preparation of ^{125}I*-labeled α-rANP*

Alpha-rANP was chemically synthesized by the solid phase method [12]. Iodination of the peptide was carried out by the lactoperoxidase method, and monoiodinated α-rANP ([^{125}I]α-rANP) was purified by C_{18} reverse phase HPLC [13]. The specific activity of [^{125}I]α-rANP was 600 μCi/μg. Equipotency of nonradioactive I-α-rANP to unlabeled α-rANP was confirmed by the chick rectum relaxation assay [14].

Preparation of samples

Renal glomeruli and tissue homogenates. Male Sprague–Dawley rats (9- to 10-weeks-old) were anesthetized with pentobarbital (65 mg/kg), and kidneys, lungs and adrenal glands were dissected. Lungs were also obtained from mongrels. Renal glomeruli were isolated from dissected kidneys by the mesh method [15]. Purity was evaluated, and the number of isolated glomeruli was counted by light microscopy. Capsules were isolated from adrenal glands using forceps. Adrenal capsules and lung were then homogenized with a Polytron homogenizer (Kinemetica, Switzerland) for 30 sec × 2 at a setting of 0.7 in 6 vol. of 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation of the homogenate at 200 *g* for 10 min and at 600 *g* for 10 min, cell debris was discarded and the supernatant fraction was saved for binding assays.

Crude membranes from renal glomeruli and lungs. Glomeruli were suspended in 12 vol. of buffer

[10 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF, 0.4 mg/ml soybean trypsin inhibitor (SBTI), pH 7.5] and homogenized for 2 min (30–40 strokes) in a motor-driven Teflon/glass homogenizer. The supernatant fraction after centrifugation at 800 *g* for 10 min was centrifuged again at 100,000 *g* for 60 min, and the pellet was resuspended in the above buffer and saved. Crude membranes were prepared from lung homogenates in a similar fashion.

Partially purified membranes. Crude membrane fractions of renal glomeruli and lungs were layered on 32% (w/w) sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, and were centrifuged at 100,000 *g* for 100 min. ANP binding activity accompanied the membrane fraction left at the top of the tube. The fraction was spun down, washed, and resuspended in homogenizing buffer.

Solubilized membrane preparations. A crude membrane fraction from renal glomeruli was spun down at 30,000 *g* for 20 min and resuspended in a solubilizing buffer [1% (w/v) Triton X-100, 20 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4]. The suspension was placed on ice for 30 min and then centrifuged at 100,000 *g* for 30 min. Four volumes of an incubation buffer [10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose, 0.2% (w/v) bovine serum albumin (BSA), 1 mM PMSF, 0.4 mg/ml SBTI] was added to the supernatant fraction and stored at −80°.

ANP receptor assay

An aliquot (0.25 ml) of glomerulus suspension or tissue homogenate (lungs and adrenal capsules) was incubated with 4–5 × 10^{-10} M [^{125}I]α-rANP (equivalent to *ca.* 3 × 10^5 cpm) in the presence or absence of 1.7–5.0 × 10^{-7} M unlabeled α-rANP at 0° or 25°. Non-specific binding was estimated in the presence of an excess concentration of unlabeled α-rANP. Reversibility of the binding of [^{125}I]α-rANP to its receptor was evaluated by adding over 1000-fold excess concentrations of unlabeled α-rANP at various time points during incubation. At the end of the incubation, 1 ml of ice-cold incubation buffer was added and 1-ml aliquots were immediately centrifuged at 10,000–15,000 *g* for 5 min. The radioactivity associated with the pellet was counted for 1 min in a γ-spectrometer.

Partially purified membranes from lungs or solubilized glomerular membranes (125 μl) were incubated with 4–5 × 10^{-10} M [^{125}I]α-rANP (*ca.* 1.5 × 10^5 cpm) in the presence or absence of 1 × 10^{-6} M unlabeled α-rANP. Bound/free ligand separation was carried out by the addition of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5% (w/v) γ-globulin (Cohn fraction II) and 0.04% (w/v) NaN_3 to 125 μl of reaction mixture, which was then vortexed with 0.25 ml of 30% (w/v) polyethylene glycol 6000. After standing on ice for 15 min, aliquots (0.5 ml) were centrifuged at 15,000 *g* for 15 min. Radioactivity bound to the pellets was counted in a γ-spectrometer.

HPLC analysis of the radioactive material bound to renal glomeruli and renal membranes

Renal glomeruli and partially purified membranes from glomeruli were incubated with [^{125}I]α-rANP (4 × 10^{-10} M) for either 10 min at 25° or for 30 min at 0°. At the end of incubation, excess unlabeled α-rANP was added. Immediately after separation of bound and free hormone by centrifugation, acetic acid was added to the supernatant fraction to a final concentration of 1 N. The pellet was transferred to a siliconized test tube (13 × 100 mm, Corning, NY) which contained 0.3 ml of boiling 1 N CH_3COOH. The test tubes were boiled for 10 min on a heating block. After centrifugation at 8000 *g* for 5 min, the radioactivity of the supernatant fraction was counted, and an aliquot was chromatographed on a Cosmosil 5 C_{18}-P column (4.6 × 100 mm, Nakarai Chemicals, Kyoto, Japan) with authentic non-radioactive markers (α-rANP, I-α-rANP and I_2-α-rANP). Elution conditions are described in the legend to Fig. 2. Elution of peptide was monitored by absorbance at 210 nm, and the radioactivity was measured for each 1- or 2-ml fraction.

Determination of cGMP response to α-rANP in renal glomeruli

Renal glomeruli were incubated with α-rANP in Krebs–Henseleit solution (118 mM NaCl, 4.76 mM KCl, 2.54 mM $CaCl_2$, 2.44 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.55 mM dextrose) containing 1 mM 3-isobutyl-1-methyl xanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterase. The concentration of cGMP induced by the irreversibly bound α-rANP was measured utilizing 125 μl of glomerulus suspension which was incubated with 5 × 10^{-7} M α-rANP at 0° for 20 min. After centrifugation at 6000 *g* for 5 min at 0°, the pellet was washed once with 125 μl of α-rANP solution (5 × 10^{-7} M) or Krebs–Henseleit solution, and the suspension was incubated at 25° for 1 min. The reaction was terminated by the addition of an equal volume of 6% (w/v) perchloric acid to the reaction mixture. Supernatant fractions from centrifugation at 3000 *g* for 10 min were adjusted to pH 3 by the addition of 60% (w/v) KOH. After centrifugation at 3000 *g* for 10 min, the supernatant fraction was succinylated, and cGMP concentration was determined using a cGMP radioimmunoassay kit ([16]; Yamasa, Chiba, Japan).

Protein determination

Protein concentration was determined by the method of Bradford [17] using γ-globulin as a standard.

RESULTS

Two distinct modes of binding of α-rANP to its receptor

Homogenates of adrenal capsules were incubated with 5 × 10^{-10} M [^{125}I]α-rANP at 0° or 25°, and reversibility of the binding of the radioligand was assessed by adding 25 μl of unlabeled α-rANP to 225 μl of reaction mixture (final concentration = 5 × 10^{-7} M) at various time points (Fig. 1). Addition of excess cold ligand 2 min after the onset of incubation at 0° lowered the bound radioactivity rapidly (within 1 min) to a level which was higher than the nonspecific binding. The reduced level was constant during incubation for a further 40 min (Fig. 1a). We

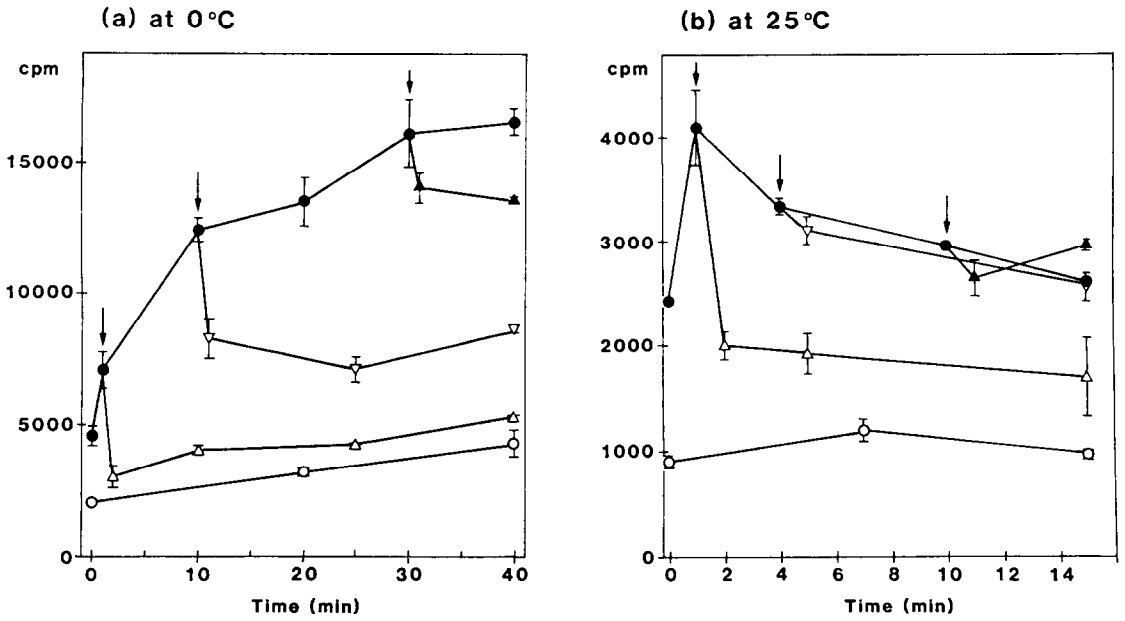

Fig. 1. Time course of [^{125}I]α-rANP binding to homogenates of adrenal capsules. Homogenates of adrenal capsules were incubated with [^{125}I]α-rANP (5×10^{-10} M) at 0° (a) or at 25° (b). Unlabeled α-rANP (5×10^{-7} M) was added to the reaction mixture at the times indicated by the arrows. Protein content/tube was (a) 252 μg and (b) 142 μg. Key: (●) total binding; (○) nonspecific binding; and (△, ▽, ▲) irreversible binding.

define the competitively released [^{125}I]α-rANP as the reversibly bound α-rANP, and [^{125}I]α-rANP remaining bound to the receptor after the addition of 1000-fold excess unlabeled α-rANP as the irreversibly bound α-rANP. The ratio of the irreversible binding to the total specific binding increased with time, suggesting that the binding of α-rANP to its specific receptor undergoes a change from a reversible to an irreversible state. This change was temperature dependent and was completed within 4 min at 25° (Fig. 1b), whereas it was not by 30 min at 0° (Fig. 1a).

A similar change was also observed in lungs and renal glomeruli (Table 1). Binding of [^{125}I]α-rANP to lung receptors reached a maximum after 30 min at 0°, and within 1 min at 25°. Addition of 1000-fold excess of the unlabeled ligand decreased the bound radioactivity within 1 min, and no further decrease

Table 1. Percentage of irreversible binding of [^{125}I]α-rANP in lung and renal glomerular preparations

Tissue	Preparation	Animal	Incubation temperature	5 mM NaN_3	Time to maximum binding	Irreversible binding	
						Time	Percent
Lungs	Homogenates	Rat	0°	–	30 min	10 min	63*
			25°	–	<1 min	1 min	51†
						4 min	76‡
						12 min	94§
	Partially purified membranes	Dog	25°	–	<1 min	1 min	29
						5 min	62
Renal glomeruli	Crude membranes	Rat	0°	–	ND‖	20 min	50
			37°	–	<1 min	5 min	83
	Solubilized membranes	Rat	0°	–	10 min	10 min	0
			25°	–	<1 min	7 min	81
	Intact glomeruli	Rat	0°	–	60–120 min	1 min	42
						3 min	68
						30 min	83
			0°	+	ND	30 min	100

Preparations from lungs and renal glomeruli were incubated with [^{125}I]α-rANP (5×10^{-10} M). Unlabeled α-rANP (5×10^{-7} M) was added at the times indicated, and the irreversible binding of [^{125}I]α-rANP was determined as described in Materials and Methods. Data were expressed as the percentage of the irreversible binding of the total specific binding at the same time. The specific binding of [^{125}I]α-rANP to all preparations except solubilized membranes was about 5% of the added [^{125}I]α-rANP (see Materials and Methods). Ten to twenty percent of [^{125}I]α-rANP was found specifically bound to solubilized membranes from renal glomeruli.

*–§ Total specific binding; * 13,500 cpm; † 13,800 cpm; ‡ 11,300 cpm; and § 12,100 cpm. ‖ Not determined.

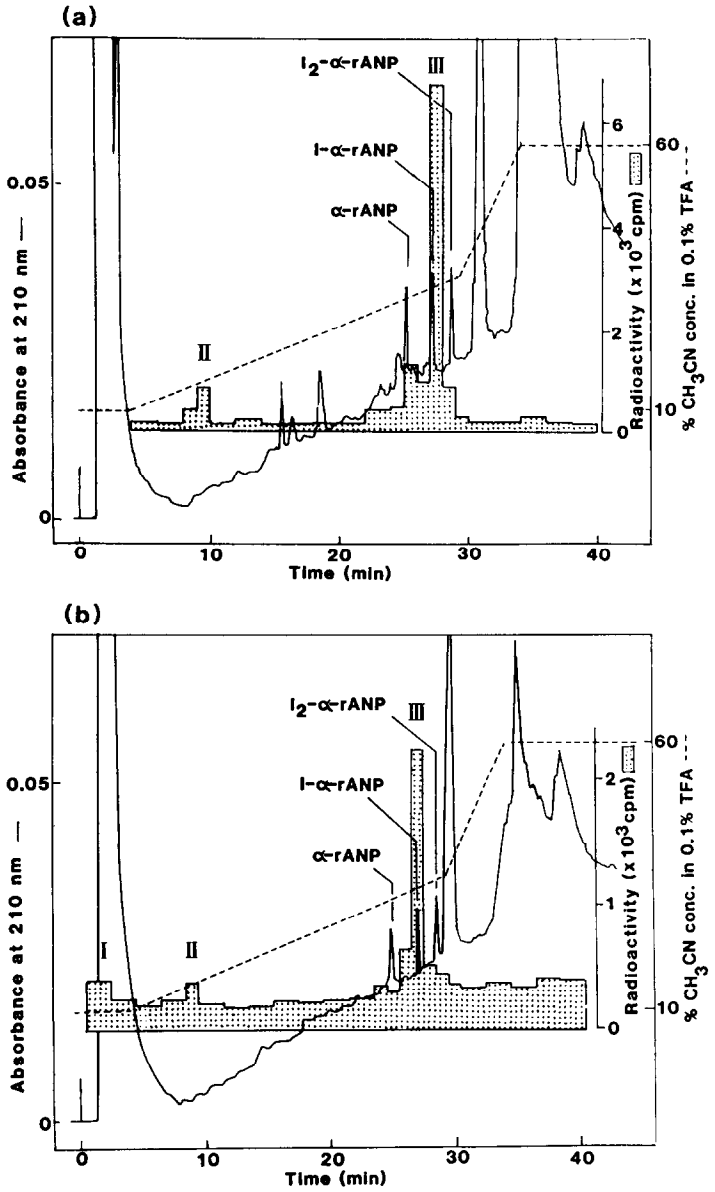

Fig. 2. Analysis of radioactive material bound to renal glomeruli by HPLC. Renal glomeruli were incubated with 4×10^{-10} M [^{125}I]α-rANP at 25° for 10 min. A 1 μM concentration of unlabeled α-rANP and 4 vol. of ice-cold incubation buffer were added, and bound and free radioactivity were separated. After centrifugation, supernatant fractions and pellets were boiled in 1 N CH_3COOH. The extracts were applied to an HPLC C_{18} reverse phase column with nonradioactive markers. I-α-rANP represents α-rANP in which 1 mol of iodine has been incorporated at ^{28}Tyr; I_2-α-rANP has 2 mol of iodine incorporated at the same amino acid residue. Retention times are shown on the abscissa, and absorption at 210 nm (A_{210}) (solid curve) and radioactivity (bar graph) are shown on the ordinate. (a) Free radioactive material recovered from the reaction mixture. (b) Radioactive material which was irreversibly bound to glomeruli. I–III indicate radioactive peaks. Elution conditions were as follows. Flow rate was 1.0 ml/min. Solvent used was: 0–4 min, 10% CH_3CN in 0.1% trifluoroacetic acid (TFA); 4–29 min, a linear gradient from 10% to 35% CH_3CN in 0.1% TFA; 29–34 min, a linear gradient from 35% to 60% CH_3CN in 0.1% TFA; 34 min-, 60% CH_3CN in 0.1% TFA.

was observed upon further incubation for 20 min at 0° or 10 min at 25° (data not shown). The irreversible binding of α-rANP in lung homogenates increased with time and was completed after 12 min at 25°. The ratio of the irreversible binding to the specific binding was significantly lower at 0° than at 25°. In addition to tissue homogenates, a similar transformation of receptor binding was also observed with a partially purified membrane preparation of canine lung. Binding of [^{125}I]α-rANP to canine lung membranes reached a plateau within 1 min at 25°. The percentage of irreversible binding to total specific binding was

29% at 1 min, but it increased to 62% by 5 min. Irreversible binding of [^{125}I]α-rANP was also observed in both intact and solubilized membranes of rat glomeruli. Irreversible binding was 50% after 20 min of incubation at 0° and 83% after 5 min at 37° using intact membranes, and 0% after 10 min at 0° and 81% after 7 min at 25° using solubilized membranes. Similarly, there were both reversible and irreversible binding to intact renal glomeruli at 0° and 25°. Irreversible binding increased from 42% at 1 min to 68% at 3 min at 0°, and it also occurred in the presence of 5 mM NaN_3.

In all cases the decrease of radioactivity upon addition of excess cold ligand was very rapid (within 1–5 min), and the irreversible radioactivity was not affected by further incubation for up to 2.5 hr.

Irreversibly bound radioactivity derived from intact [^{125}I]α-rANP

We next determined whether the irreversibly bound radioactivity was associated with intact, degraded or covalently coupled [^{125}I]α-rANP. After [^{125}I]α-rANP was bound to intact rat glomeruli or partially purified glomerular membranes, excess unlabeled α-rANP (2500-fold excess) was added to dissociate completely the reversible radioactivity. After centrifugation, ^{125}I-labeled materials were extracted with 1 N CH_3COOH from supernatant fractions and pellets. The recovery (the extracted radioactivity versus the irreversibly bound radioactivity) was 76% from glomeruli and 99% from glomerular membranes.

When unbound and irreversibly bound radioactive materials from rat glomeruli were examined using reverse phase HPLC, two radioactive peaks were found in the unbound fraction (Fig. 2a, II and III), whereas there were three radioactive peaks in the bound materials (Fig. 2b, I, II and III). By comparing with authentic standards, peak III was identified to be [^{125}I]α-rANP, peak I was free Na^{125}I, and peak II was not identified. Most of the irreversible radioactivity was associated with the I-α-rANP peak (Fig. 2b), indicating that the majority of the irreversibly bound radioactivity is associated with the native hormone. The recovery of the radioactivity after HPLC was 73% (Fig. 2b). It should also be noted that there was no significant radioactivity in high molecular weight regions. This finding suggests that α-rANP did not form an S—S linkage with proteins containing a Cys residue as has been shown in the case of α-human ANP [18]. Similar results were obtained using partially purified glomerular membranes (data not shown).

Induction of cGMP response by irreversibly bound α-rANP

When [^{125}I]α-rANP bound glomeruli were washed (Table 2, row C), binding decreased to approximately the same level as the irreversible binding measured in the presence of excess cold α-rANP (Table 2, row B). We then examined which form of the α-rANP–receptor complex is responsible for the α-rANP-mediated increase in cGMP concentrations in intact glomeruli. The irreversibly bound α-rANP clearly increased cGMP concentrations in glomeruli (Fig. 3, b), whereas there was no further increase in cGMP concentrations by a mixture of irreversibly and reversibly bound α-rANP (Fig. 3, a).

Table 2. Release of reversibly bound [^{125}I]α-rANP by unlabeled α-rANP or by washing

	[^{125}I]α-rANP (cpm)*
A Total binding	4328 ± 662
B Irreversible binding†	2940 ± 375
C Binding after wash‡	2957 ± 403
D Nonspecific binding	1309 ± 121

Rat renal glomeruli were incubated in 0.25 ml with [^{125}I]α-rANP (3.3×10^{-10} M) for 20 min at 0°. The mixture was centrifuged, and pellets were resuspended in 0.25 ml of unlabeled α-rANP (5×10^{-7} M) or in incubation buffer (see Materials and Methods). Then bound radioactivity was counted by the procedure described in Materials and Methods. The number of glomeruli in one tube was approximately 1500 (purity 79%).

* Mean ± SD (N = 3).

† Centrifuged and resuspended in 5×10^{-7} M unlabeled α-rANP.

‡ Centrifuged and resuspended in 0.25 ml of buffer solution.

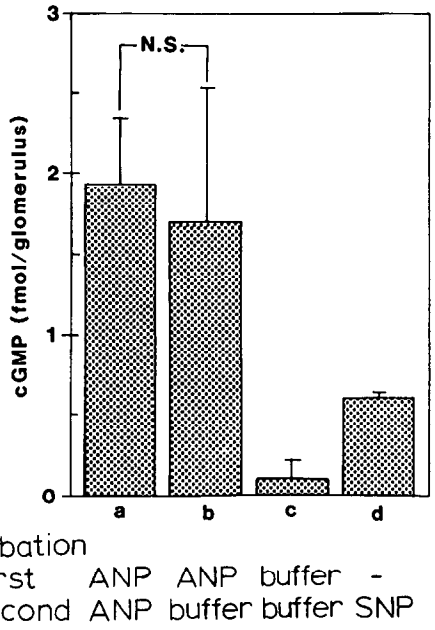

Fig. 3. Reversible and irreversible binding of α-rANP and cGMP response. Glomeruli were first incubated with 5×10^{-7} M α-rANP (a, b) or Krebs–Henseleit solution (c) at 0° for 20 min. After centrifugation, glomeruli were washed with Krebs–Henseleit solution (a, b, c) and resuspended in 5×10^{-7} M α-rANP (a) or in the buffer solution (b, c) in the original volume. They were then incubated at 25° for 1 min. Column (d) is for a 1-min incubation at 25° with 0.1 mM sodium nitroprusside (SNP), an activator of soluble guanylate cyclase. The bar graph indicates the amount of cGMP produced during incubation at 25° for 1 min. All reaction mixtures contained 1 mM IBMX. The number of glomeruli/tube was 960 ± 170 (purity 71%). N.S. = not significant.

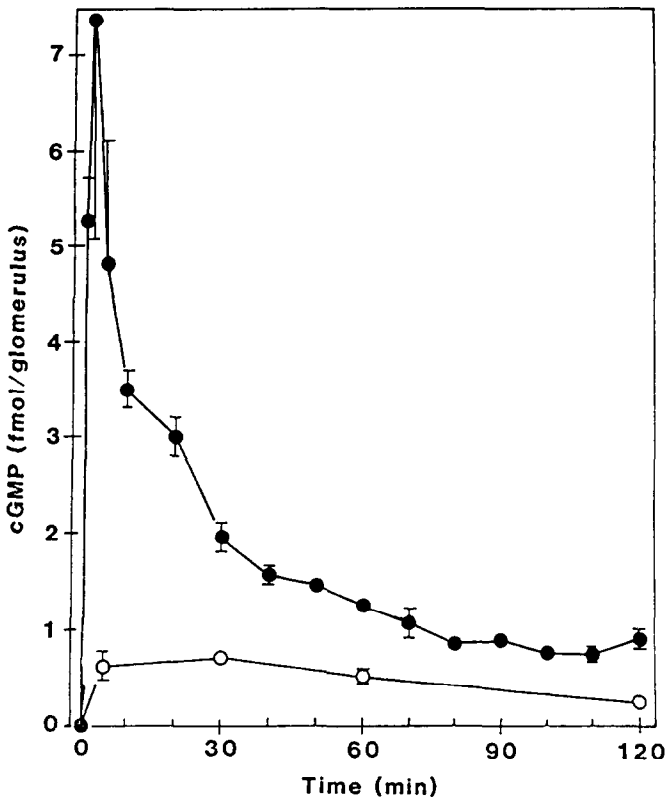

Fig. 4. Changes in cGMP content in renal glomeruli during incubation with α-rANP. Renal glomeruli were incubated at 25° with (●) or without (○) 9×10^{-7} M α-rANP, and changes of cGMP content were measured. All reaction mixtures contained 1 mM IBMX. The number of glomeruli/tube was 1853 ± 287 (purity = 70%).

When renal glomeruli were incubated with α-rANP, cGMP content reached a maximum at 2 min and decreased thereafter (Fig. 4). It should be noted that a rapid decrease in cGMP concentration occurred despite the presence of 1 mM IBMX, an inhibitor of phosphodiesterase. To examine the change in guanylate cyclase activity during incubation, renal glomeruli were incubated with α-rANP (5×10^{-7} M), and 1 mM IBMX was added at 0, 5 and 20 min. An increase in cGMP concentration in response to α-rANP was observed at all time points (Fig. 5), suggesting that α-rANP activates guanylate cyclase during incubation for at least 20 min.

DISCUSSION

We have distinguished reversible and irreversible binding of [^{125}I]α-rANP to its receptor by competitive displacement of the bound radioligand. Addition of 1000-fold concentration of unlabeled ligand decreased the bound radioactivity very rapidly to a constant level which did not show further loss of radioactivity upon incubation. The results of this study show that the binding of α-rANP to its receptor undergoes transformation from a reversible to an

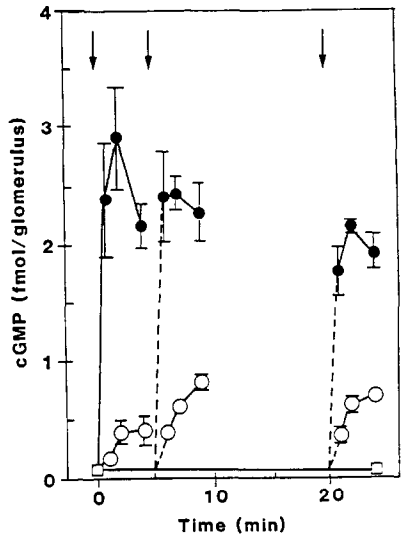

Fig. 5. Activation of guanylate cyclase activity by α-rANP. Renal glomeruli were incubated at 25° with (●) or without (○) 5×10^{-7} M α-rANP. At 0, 5 and 20 min, 1 mM IBMX was added. Key: (□) none. The number of glomeruli/tube was 847 ± 186 (purity 71%).

irreversible state in preparations such as intact renal glomeruli, homogenates of adrenal capsules and lungs, membranes of glomeruli and lungs, and solubilized glomerular membranes. Furthermore, our data show that the irreversibly bound α-rANP was able to increase cGMP concentration, presumably by stimulating guanylate cyclase activity. It should also be noted that two types of ANP receptors are known. One is thought to be coupled to the membrane-bound guanylate cyclase, while the other is not [9, 19]. Whether the observed differential binding of α-rANP is related to different types of ANP receptors is not currently known.

Since it has been suggested that a receptor-bound α-rANP may become internalized at 37° in intact cells [20], it is important to distinguish irreversible binding from internalization of α-rANP. Our data demonstrating the appearance of the irreversibly bound α-rANP in disrupted cell preparations precludes the possibility of ligand–receptor internalization. A significant amount of irreversible binding was also found in glomeruli in the presence of 5 mM NaN_3 at 0°, suggesting that the formation of an irreversible α-rANP–receptor complex is independent of an energy-requiring process typically involved in the receptor internalization. Thus, transformation of reversible to irreversible binding of α-rANP to its receptor reflects a change in the mode of interaction within the same α-rANP–receptor complex. In this respect, it is interesting to note that there are two distinct states (high affinity binding and low affinity binding) in the interleukin-2 receptor (p55 [Tac antigen]) for its ligand and that the high affinity state is the one that is involved in signal transduction [21].

Our findings suggest that the appearance of irreversible binding of α-rANP to its receptor may be involved in signal transduction and in subsequent biological responses. Specifically, the transformation of the reversible to the irreversible α-rANP-receptor may be a critical event in the activation of guanylate cyclase which is thought to be the initial response to ANP in target tissues [8–11].

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