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Binding of synthetic β-human atrial natriuretic peptide to cultured rat vascular smooth muscle cells

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We have studied the effects of synthetic β-human atrial natriuretic peptide (β-hANP), an antiparallel dimer of α-hANP, on receptor binding and cGMP generation in cultured rat vascular smooth muscle cells and compared the effects with those of α-hANP. Characteristics of temperature-dependent binding and degradation of ^{125}I-β-hANP were similar to those of ^{125}I-α-hANP. Scatchard analysis indicated a single class of binding sites for β-hANP with a maximal binding capacity one-half that of α-hANP. Parallel and antiparallel dimers were equipotent in inhibiting the binding and stimulating intracellular cGMP formation, of which the maximal effect was about one-half that of α-hANP. Reverse-phase high performance liquid chromatography revealed that most of β-hANP added to cells was converted to a small molecular mass component corresponding to α-hANP after incubation. These data suggest that the less potent effect of β-hANP in receptor binding and cGMP generation may be partly accounted for by the possible conversion of β-hANP to α-hANP at the site of target cells.

Atrial natriuretic peptide; Receptor binding; cGMP; (Vascular smooth muscle cell, Human)

1. INTRODUCTION

In human atria, three distinct forms of human atrial natriuretic peptide (hANP) have been identified [1,2]: α (28 residues), β (56 residues), and γ (126 residues). α-hANP is the major cardiac hormone with a potent natriuretic and vasoactive activity [1], while γ-hANP having the sequence of α-hANP at its C-terminus, is a precursor molecule for α-hANP [2,3]. In contrast, β-hANP comprises an antiparallel dimer of α-hANP [2], although whether such a dimetric peptide processing occurs in vivo remains unknown. Recently, β-hANP has been synthesized and its biological activity has been determined [4,5]. Interestingly, natural [2] as well as synthetic β-hANP [4,5] exhibits a slower onset and longer duration of action than α-hANP, the mechanism of which remains obscure.

Using cultured rat vascular smooth muscle cells (VSMCs) which have specific receptors for ANP functionally coupled to the guanylate cyclase system [6], we have clarified the cellular mechanism of binding, internalization and degradation of α-rat (r) ANP [7]. To elucidate the mechanism by which β-hANP acts on the target cell, we have studied the effects of synthetic β-hANP on receptor binding and cGMP generation in cultured rat VSMCs in comparison with that of α-hANP, and examined the change in molecular size of β-hANP coincubated with the cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Antiparallel (β-hANP) and parallel dimers of α-hANP were synthesized by a conventional solution method as reported [4]. Synthetic α-hANP was also obtained from Peptide Institute (Osaka, Japan). Iodination of α-hANP and β-hANP was performed by the lactoperoxidase method [6] with specific activities of ~150 μCi/μg and ~50 μCi/μg, respectively.

2.2. *Binding experiment*

Rat aortic VSMCs were cultured and used in the experiments as reported [6]. Confluent cells ($0.5-1 \times 10^6$ cells) were incubated with ^{125}I-α-hANP or ^{125}I-β-hANP in Hanks' balanced salt solution, pH 7.4, containing 0.1% bovine serum albumin (binding medium) at 4°C for 3 h to minimize internalization and/or degradation of radioligand. After completion, cells were washed, solubilized with NaOH, and cell-bound radioactivity was determined. Specific binding was defined as total binding minus nonspecific binding in the presence of excess (3.2×10^{-7} M) unlabeled α-hANP or β-hANP.

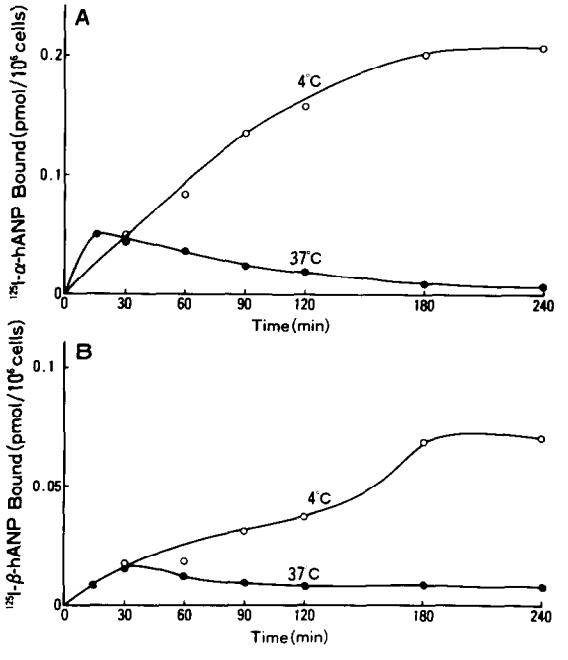

Fig.1. Time course of specific binding of ^{125}I-α-hANP and ^{125}I-β-hANP to cultured rat VSMCs at 37°C and 4°C. Confluent cells (10^6 cells) were incubated with 2 nM ^{125}I-α-hANP (A) and ^{125}I-β-hANP (B) at 37°C (●) or 4°C (○). At the indicated times, specific cell-bound radioactivity was determined; each point is the mean of triplicate dishes.

2.3. *Internalization and degradation*

To study degradation, the cells were preincubated with radioligands at 4°C for 3 h, rinsed, and then incubated at either 37°C or 4°C; the cell-bound radioactivity was determined and trichloroacetic acid-insoluble radioactivity was also measured in aliquots of the medium. For analysis of internalization, prebound radioligand was dissociated with 0.1 M acetic acid treatment that only extracts surface-bound, but not internalized ligands [7,8].

2.4. *Determination of intracellular cGMP*

VSMCs were incubated with or without various doses of α-hANP or β-hANP at 37°C for 10 min in binding medium containing 0.5 mM methylisobutylxanthine [6]. Intracellular cGMP were determined by radioimmunoassay (RIA).

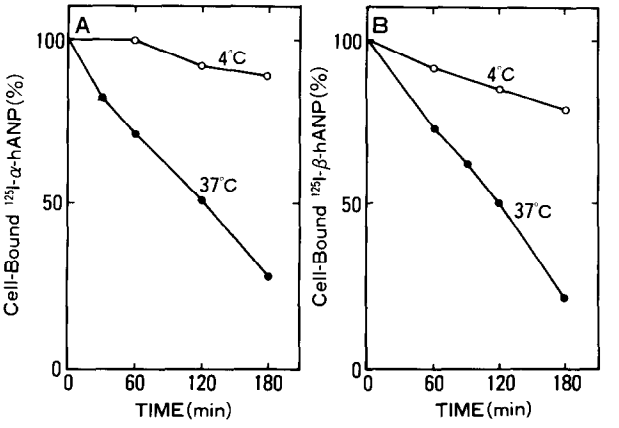

Fig.2. Time course of decrease of cell-bound ^{125}I-α-hANP and ^{125}I-β-hANP from cultured rat VSMCs at 37°C and 4°C. After preincubation with 1 nM ^{125}I-α-hANP (A) and ^{125}I-β-hANP (B) at 4°C for 3 h, cells were further incubated in fresh medium at 37°C (●) or 4°C (○). At the indicated times, the cell-bound radioactivity was determined. The results are expressed as the percentage of binding at indicated time intervals to initial binding; each point is the mean of duplicate dishes. Specific binding of ^{125}I-α-hANP and ^{125}I-β-hANP at zero time was 85% and 50% of total binding, respectively.

2.5. *Chromatographic analysis*

To examine whether the change in molecular size of exogenous β-hANP occurs during incubation, the media in which β-hANP was coincubated with or without VSMCs at 37°C for 60 min were applied to a Sep-Pak C_{18} cartridge (Waters Associate, Milford, MA) followed by washing with distilled water and elution with 5 ml of 86% ethanol/4% acetic acid. The eluate was evaporated to dryness under nitrogen gas and subjected to reverse-phase high pressure liquid chromatography (HPLC) on a 4.6 × 250 mm Zorbax ODS column (DuPont Co., Wilmington, DE) with a linear gradient from 10 to 60% acetonitrile for 80 min. The flow rate was 1 ml/min; 1-ml fractions were collected. The eluates were dried and assayed for hANP by RIA as described in [9]. α-hANP RIA was performed using rabbit anti-α-hANP antiserum that showed 50% cross-reactivity with β-hANP on a molar basis.

3. RESULTS

Binding of ^{125}I-α-hANP and ^{125}I-β-hANP to cultured rat VSMCs showed almost similar temperature- and time-dependency (fig.1); at 37°C binding of both radioligands rapidly reached to a maximum at 15–30 min and decreased thereafter, while their maximal binding at 4°C was slow (3–4 h) but about 3–4-fold greater than that at 37°C. Specific binding of ^{125}I-α-hANP and ^{125}I-β-hANP was 90–95% and 50–60% of total binding, respectively. The prebound radioactivity of either ^{125}I-α-hANP or ^{125}I-β-hANP at 4°C, after washing, decreased more rapidly upon warming the cells to 37°C than further incubation at 4°C (fig.2); most (>80%) radioactive material released into medium at 37°C was trichloroacetic acid-soluble, while at 4°C it was exclusively trichloroacetic acid-insoluble. Furthermore, about one-third of cell-bound ^{125}I-α-hANP and ^{125}I-β-

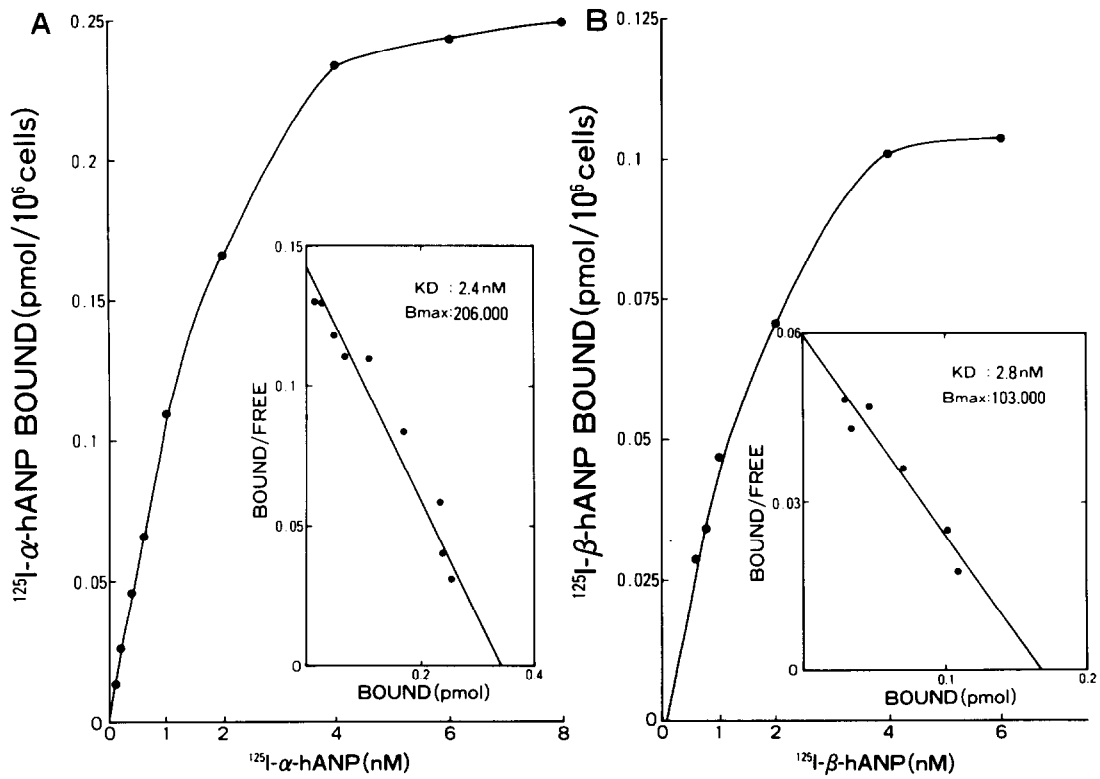

Fig.3. Saturable binding of ^{125}I-α-hANP and ^{125}I-β-hANP to cultured rat VSMCs. Confluent cells (10^6 cells) were incubated with various doses of ^{125}I-α-hANP (A) and ^{125}I-β-hANP (B) at 4°C for 3 h. Specific binding of ^{125}I-α-hANP and ^{125}I-β-hANP was 80–90% and 50–60% of total binding, respectively. Each point is the mean of two experiments. (Insets) Scatchard plots of binding data. K_D, dissociation constant; B_{max}, number of maximal binding sites per cell.

hANP at 37°C shifted to the acid-resistant compartment, whereas at 4°C they were mostly localized on the acid-extractable compartment (not shown). These experiments suggest that, after washing, the observed temperature-dependent decrease of cell-bound radioactivity is most probably due to internalization, degradation, and elimination of ligands rather than simple dissociation from the cell surface as demonstrated [7].

An equilibrium binding of ^{125}I-α-hANP and ^{125}I-β-hANP at 4°C was a saturable process (fig.3). Scatchard plots of the binding (fig.3, inset) revealed the presence of a single class of binding sites for both α-hANP and β-hANP: the apparent dissociation constants (K_d) were almost the same ($\sim 2 \times 10^{-9}$ M), while the maximal binding sites (B_{max}) of β-hANP (103000 sites/cell) was half that

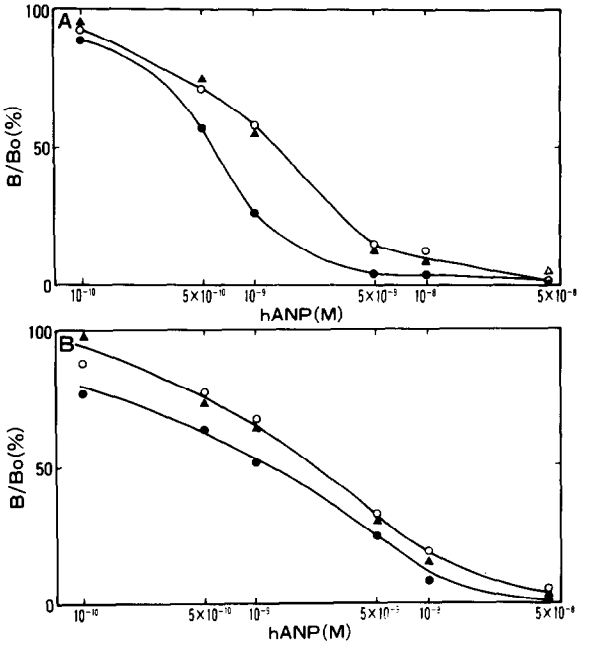

Fig.4. Competitive binding of ^{125}I-α-hANP and ^{125}I-β-hANP to cultured rat VSMCs by unlabeled α-hANP and β-hANP. Confluent cells (5×10^5 cells) were incubated at 4°C for 3 h with 1 nM ^{125}I-α-hANP (A) and ^{125}I-β-hANP (B) in the absence and presence of various doses of unlabeled α-hANP (●), its antiparallel (○) and parallel (▲) dimers. Results are expressed as the percentage to specific binding in the absence of peptides (B_o); each point is the mean of duplicate dishes. Specific binding of ^{125}I-α-hANP and ^{125}I-β-hANP was 98% and 67% of total binding, respectively.

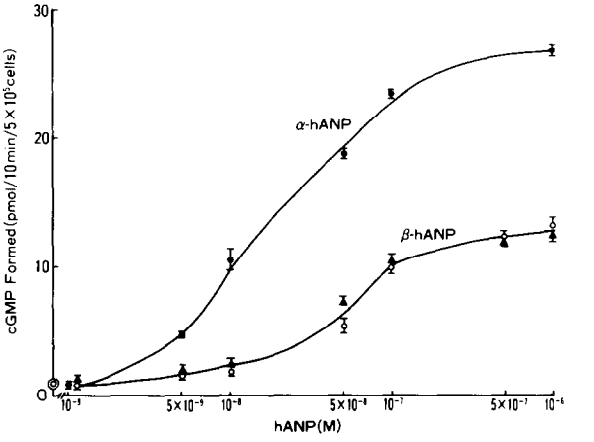

Fig.5. Effects of α-hANP and β-hANP on formation of intracellular cGMP in cultured rat VSMCs. Confluent cells (5×10^5 cells) were incubated at 37°C for 10 min in the absence (◎) and presence of various doses of α-hANP (●), its antiparallel (○) and parallel (▲) dimers. Concentrations of intracellular cGMP were determined by RIA. Each point is the mean of three samples: bar shows SE.

of α-hANP (206000 sites/cell). Competitive binding of ^{125}I-α-hANP (fig.4A) and ^{125}I-β-hANP (fig.4B) by unlabeled α-hANP and its dimers was studied; parallel and antiparallel dimers equally inhibited the binding of both radioligands with almost the same IC_{50} ($\sim 1-2 \times 10^{-9}$ M), but they were about 2-fold less potent than α-hANP in

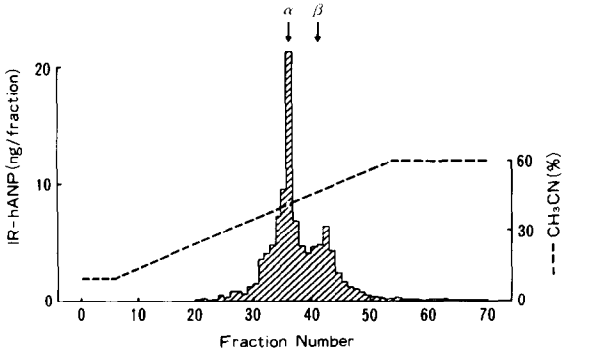

Fig.6. An elution profile of reverse-phase HPLC. The medium in which β-hANP was coincubated with VSMCs at 37°C for 60 min was subjected to a reverse-phase HPLC. Concentrations of immunoreactive (IR) hANP were determined. Arrows denote the elution positions of standard α-hANP (α) and β-hANP (β), respectively.

displacing both radioligands from their binding sites.

As shown in fig.5, parallel and antiparallel dimers were equipotent in stimulating cGMP formation with an approximate EC_{50} ($\sim 5 \times 10^{-8}$ M) comparable to that of α-hANP, whereas the maximal stimulation on cGMP generation by two dimers was about one-half that by α-hANP.

Reverse-phase HPLC of the medium in which β-hANP was coincubated with cells revealed two immunoreactive (IR) peaks (fig.6); a major peak comprising about two-third of total immunoreactivity emerged at the elution position of standard α-hANP, while a minor peak comigrated with standard β-hANP. When β-hANP was incubated in the same medium without cells, only the single IR peak coeluting with standard β-hANP was observed (not shown).

4. DISCUSSION

It has recently been shown that synthetic β-hANP, an antiparallel dimer of α-hANP, has biological properties similar to natural β-hANP [4,5]. The present study first demonstrates that synthetic β-hANP binds to cultured rat VSMCs in the same manner as α-hANP in terms of time- and temperature-dependency. Furthermore, the present binding experiments suggest that both α-hANP and β-hANP initially bound to cell-surface membranes are similarly internalized, degraded, and subsequently released into medium in a temperature-dependent manner. In fact, degradation of cell-bound ^{125}I-β-hANP at 37°C was significantly blocked by pretreatment with lysosomotrophic drugs (NH_4Cl, chloroquine) and an autoradiographic study using electron microscopy revealed that grains of ^{125}I-β-hANP were localized not only on the cell membrane, but also within lysosome-like structures at 37°C (unpublished observations). Taken together, our data are consistent with the cellular mechanism of binding, internalization and degradation of β-hANP similar to that of α-rANP as we have demonstrated [7]. Therefore, it seems unlikely that altered binding affinity and/or delayed degradation of β-hANP may be responsible for the observed slower onset and longer duration of action by β-hANP [2,4,5].

While we have reported that only a small quantity (2–3%) of β-hANP is converted to α-hANP under in vitro conditions [4], the present study clearly shows that most of the β-hANP added to VSMCs in a serum-free condition is eventually converted to a small molecular sized component corresponding to α-hANP, suggesting the possible conversion from dimeric into monomeric form during incubation. Therefore, it seems likely that the observed slower onset and longer duration of β-hANP action [2,4,5] may be in part accounted for by the conversion of β-hANP to α-hANP at the site of target organs and/or in the circulation. However, whether the cleavage of intermolecular disulfide bonds of β-hANP and reformation of an intramolecular disulfide bond of α-hANP involve cell-mediated enzymatic processing remains to be determined.

In the present studies, maximal binding capacity of β-hANP is shown to be one-half that of α-hANP, while the apparent binding affinity is almost the same. This is in accord with our result that the maximal stimulatory effects of intracellular cGMP formation by parallel and antiparallel dimers, although almost identical with one another, appear to be about half that by α-hANP. These data are also compatible with those of biological activities of β-hANPs [4,5]; parallel and antiparallel forms are equipotent but equally less potent than α-hANP in smooth muscle spasmolytic in vitro assays as well as natriuretic in vivo assays. Assuming that β-hANP is fully convertible to α-hANP that interacts with its receptor and stimulates guanylate cyclase, β-hANP should be twice as active as α-hANP on a molar basis. However, this is not the case because β-hANP bound only to half the receptor population. One would thus speculate that about one-fourth of the dimeric form may be convertible to the monomeric form to explain the less potent effect of β-hANP in receptor binding and cGMP generation. The precise mechanism by which β-hANP is converted into α-hANP in vivo remains to be elucidated.

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