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A dicarba analog of β-atrial natriuretic peptide (β-ANP) inhibits guanosine 3′,5′-cyclic monophosphate production induced by α-ANP in cultured rat vascular smooth muscle cells

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The synthesis and biological properties are described of [Asu$^{7,23'}$]-β-ANP-(7–28) (Asu, L-α-aminosuberic acid), a dicarba analog of β-atrial natriuretic peptide (β-ANP, an antiparallel dimer of human α-ANP with the chains linked by 7–23′ and 7′–23 disulfide bonds). This Asu-analog (referred to as analog III) displaced ^{125}I-α-ANP specifically bound to cultured rat vascular smooth muscle cells (VSMC) with an apparent K_i of 2.1×10^{-8} M, but did not stimulate formation of intracellular cGMP at 10^{-8}–10^{-5} M. Analog III inhibited the α-ANP-stimulated cGMP production in VSMC competitively with a pA_2 value of 7.45 and behaved as an antagonist of α-ANP in rat aorta smooth muscle relaxation. In addition, β-ANP was also shown to inhibit the α-ANP-induced cGMP production in a dose-dependent manner. The mechanism of action of β-ANP is also discussed.

Atrial natriuretic peptide analog; Atrial natriuretic peptide receptor antagonist; Receptor binding; cyclic GMP production; Vasorelaxation; (Cultured vascular smooth muscle cell)

1. INTRODUCTION

Human cardiocytes produce a potent vasorelaxant and natriuretic factor called atrial natriuretic peptide (ANP) in three distinct molecular forms, α-, β- and γ-ANP [1]. α-ANP is a 28-amino-acid peptide with an intramolecular disulfide bond and is believed to be derived from γ-ANP, which is present predominantly in the heart and comprises 126 amino acid residues including an α-ANP sequence at the C-terminal. β-ANP is an antiparallel dimer of human α-ANP, in which the two monomer chains are linked by Cys^7–Cys$^{23'}$ and Cys$^{7'}$–Cys^{23} disulfide bonds [2]. β-ANP has not been found in species other than man and its mechanism of formation as well as its physiological role remain unknown.

β-ANP is reported to exhibit slower onset and longer duration than α-ANP in diuresis and natriuresis [2–4]. β-ANP is also known to be converted into α-ANP when incubated with human plasma [5] or rat cultured vascular smooth-muscle cells (VSMC) [6]. In view of these facts, Itoh et al. [5] suggested that the slow onset and long-acting property of β-ANP was a consequence of its slow conversion into α-ANP. Recently, they have shown that β-ANP exogenously administered to healthy volunteers elicits stronger responses and longer duration than does α-ANP when compared on a molar basis. This effect has been ascribed, if not entirely, to the dimer-to-monomer conversion

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Abbreviations: Acm, acetamidomethyl; ANP, atrial natriuretic peptide; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; cGMP, guanosine 3′,5′-cyclic monophosphate; Dcb, 2,6-dichlorobenzyl; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; HPLC, high-performance liquid chromatography; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TFA, trifluoroacetic acid; VSMC, cultured vascular smooth muscle cells

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which has also been shown to occur in circulation in vivo [7]. In spite of these observations, the question as to whether β-ANP itself is biologically active is still ambiguous.

To shed more light on the nature of β-ANP, we attempted here to synthesize and to examine the biological properties of a β-ANP analog which could not be converted into the corresponding monomer under the usual physiological conditions. Since deletion of a pair of 6-residue N-terminal segments (1–6 and 1′–6′) from β-ANP had no significant effect on the biological properties of the hormone including its long-acting nature [8], we tried to synthesize a dicarba analog of β-ANP-(7–28), [Asu$^{7,23'}$]-β-ANP-(7–28) (referred to as analog III), in which an L-3-aminosuberic acid (Asu) residue was substituted for the cystine residue corresponding to Cys-7 and Cys-23′ in β-ANP (fig.2). The present investigation has now revealed that this synthetic peptide and β-ANP as well behave as antagonists of α-ANP in stimulating cGMP production in cultured rat VSMC.

2. MATERIALS AND METHODS

2.1. *Materials*

Human α-ANP and β-ANP [3] were synthesized by solution methods in these laboratories. ^{125}I-labeled human α-ANP (1800 Ci/mmol) was purchased from Amersham and aminopeptidase M from Pierce.

2.2. *Peptide synthesis*

[Asu$^{7,23'}$]-β-ANP-(7–28) (analog III) was synthesized by the conventional solution methods as illustrated in fig.1. An Asu-containing octapeptide derivative (**1**) corresponding to positions 21′–28′ of β-ANP was coupled successively with properly protected fragments 8–28, 17′–20′ and 7′–16′ by the EDC-HOBt method to give a protected derivative of analog III (**2**). Compound **2** was, after removal of the Boc group with TFA, treated with HF-anisole-dimethylsulfide to give [Asu$^{7,23'}$, Cys(Acm)$^{7'}$, Cys(Acm)^{23}]-β-ANP-(7–28) in a yield of 41%. The removal of Acm groups by treatment with $Hg(OAc)_2$ at pH 4 was followed by air-oxidation under conditions of high dilution and pH 7 to afford analog III in a yield of 32% after purification by preparative HPLC. $[\alpha]D^{23}$ −46.9° (*c* 0.1, 1 M acetic acid). The amino acid ratio in acid hydrolysate (6 M HCl, 5% thioglycolic acid, 110°C, 24 h) was Asp 3.88 (4), Ser 3.37 (4), Glu 2.01, (2), Gly 10.06 (10), Ala 2.08 (2), Met 1.95 (2), Asu 1.08 (1), Ile 1.97 (2), Leu 2.01 (2), Tyr 2.02 (2), Phe 4.00 (4), Arg 6.00 (6),

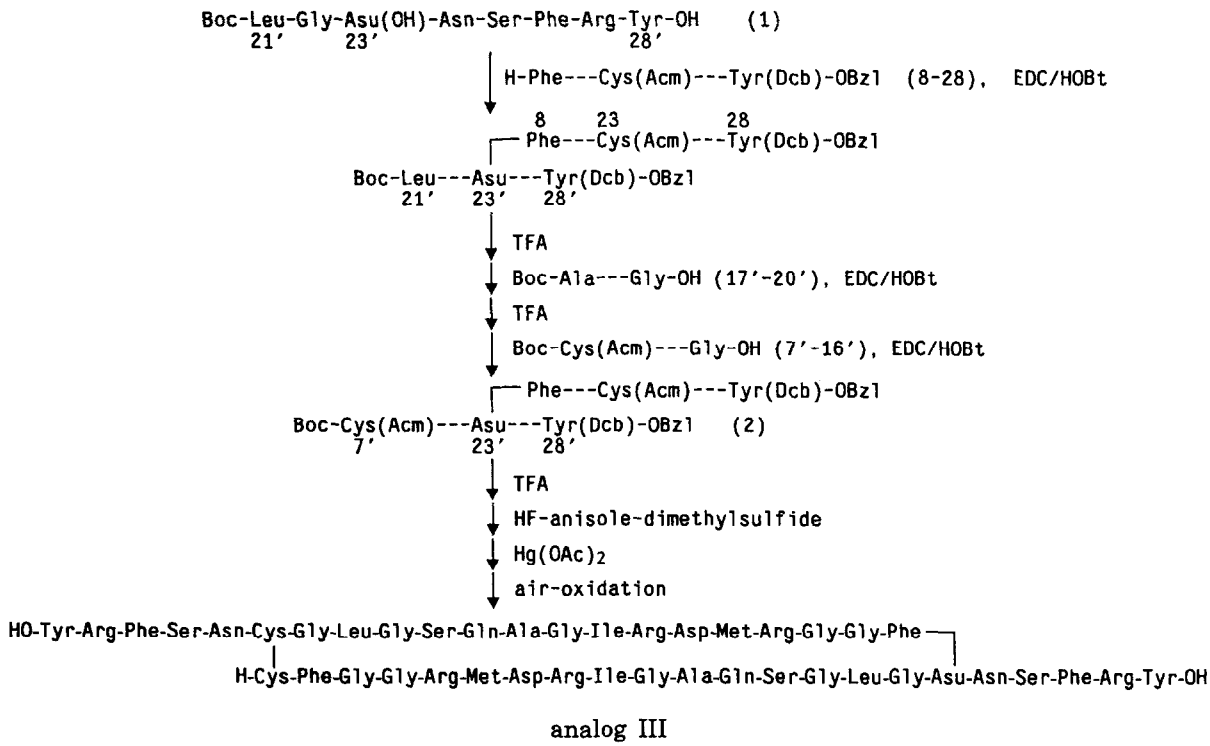


Fig.1. Scheme for synthesis of analog III.

recovery 71%. Cystine was determined on an aminopeptidase M digest: 0.91 (1).

2.3. *Culture of rat VSMC*

Vascular smooth muscle cells (VSMC) obtained from the thoracic aorta of 20-week-old male Wistar rats were cultured in virtually the same manner as described by Hirata et al. [9]. Subcultured VSMC between 6th and 10th passages were used in the present experiments.

2.4. *Binding experiments*

VSMC were seeded into 24-well plates (Falcon) and cultured to confluency. The cells were washed twice with Hank's medium containing 0.1% bovine serum albumin and 2 mM Hepes (assay medium, 0.3 ml) and then incubated with ^{125}I-α-ANP in assay medium (0.5 ml) in the presence of different concentrations of peptide at 23°C for 60 min. After removal of the incubation medium by aspiration, the cells were quickly washed twice with ice-cold assay medium (0.3 ml) and solubilized with 10% SDS (0.5 ml) and 0.5 M NaOH (0.5 ml), successively. The cell-bound radioactivity was measured in a gamma counter (Aloka ARC-360).

2.5. *Measurements of intracellular cGMP*

The confluent VSMC in 24-well plates were washed with assay medium and incubated in assay medium (0.5 ml) at 37°C for 30 min in the presence or absence of peptide. The incubation medium was aspirated off and ice-cold 6% trichloroacetic acid was added. The cells were mechanically scraped off and the mixture was centrifuged. The supernatant fluid was washed three times with aqueous ether and then lyophilized. The cGMP content of the residue was measured by a radioimmunoassay kit (Yamasa cyclic GMP assay kit). The data obtained were analyzed by Tukey's test [10].

2.6. *Assay for smooth muscle relaxant activity*

Thoracic aortae isolated from male Wistar rats (~ 300 g) were cut into ~ 2 × 20 mm spiral strips and suspended in a modified Locke-Ringer solution, containing 120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM $NaHCO_3$ and 5.5 mM glucose, at 37°C. The tissues were equilibrated for 60–90 min under a resting tension of 1.5 g and gassed with a mixture of 95% O_2 and 5% CO_2. The arterial strips were contracted with 1–2 μM $PGF_{2\alpha}$ in the presence or absence of analog III to an extent between 25 and 40% of the contraction induced by 30 mM KCl, and α-ANP was then added in a cumulative manner.

3. RESULTS

3.1. *Characterization of VSMC*

The rat VSMC culture prepared according to Hirata et al. [9] showed a so-called hill-and-valley structure characteristic of smooth muscle cells. The Scatchard analysis was applied to the saturable binding of ^{125}I-α-ANP to VSMC and gave an apparent dissociation constant (K_d) of 0.87×10^{-9} M and the number of maximum binding sites (B_{max}) of 230000 sites/cell. These values are well comparable to those reported in the literature ($K_d = 1.36 \times 10^{-9}$ M, B_{max} = 220000 sites/cell) [9]. The competition of ^{125}I-α-ANP with unlabeled α-ANP for binding to VSMC was also analyzed by the Scatchard plots to give $K_d = 1.2 \times 10^{-9}$ M and B_{max} = 289000 sites/cell (literature: $K_d = 2.4 \times 10^{-9}$ M, B_{max} = 278000 sites/cell [9]).

3.2. *Competitive binding to VSMC*

The ability of human α-ANP, β-ANP and analog III to displace ^{125}I-α-ANP specifically bound to VSMC was measured. The results are shown in fig.2. In the present experiment the K_i values are equivalent to the corresponding IC_{50} values (concentrations of peptide displacing 50% of ^{125}I-α-ANP), since [L] is much lower than K_d in the equation $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] and K_d denote the concentration and dissociation constant of ^{125}I-α-ANP [11], respectively. Therefore, the K_i values of α-ANP, β-ANP and analog III are estimated as 2.2×10^{-9}, 5.6×10^{-9} and 2.1×10^{-8} M, respectively. The relative

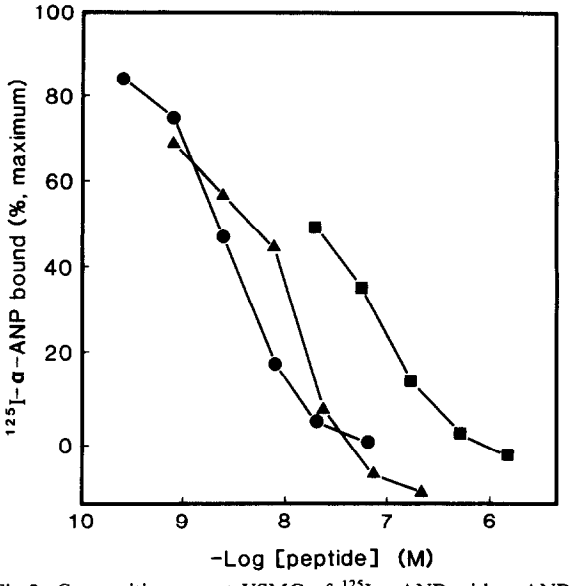


Fig.2. Competition to rat VSMC of ^{125}I-α-ANP with α-ANP and related peptides. 4.2×10^{-11} M ^{125}I-α-ANP was incubated with rat VSMC (approx. 1.3×10^{-5} cells) in the presence of various concentrations of unlabeled α-ANP (●), β-ANP (▲) or analog III (■). The experiment was performed in duplicate. Specific binding was determined as the difference between total binding and binding in the presence of 0.64×10^{-7} M unlabeled α-ANP.

potencies of β-ANP and analog III calculated as reciprocals of their IC_{50} values are 0.39 and 0.1 (α-ANP = 1), respectively.

3.4. *Effect on α-ANP-induced cGMP production*

Fig.3 shows dose-response relationships for human α-ANP, β-ANP and analog III in the production of intracellular cGMP in cultured VSMC. The production was stimulated in a dose-dependent manner by $10^{-9}-10^{-6}$ M α-ANP. Less remarkable but significant stimulation was caused by β-ANP. However, analog III did not show any stimulation even at 10^{-5} M. Then, the effect of analog III on the α-ANP-induced cGMP production was examined. In the presence of 10^{-7} M α-ANP, the intracellular cGMP level in VSMC was increased more than five times that of the control. This stimulatory effect of α-ANP was, however, diminished by 20, 50, and 80% in the presence of 10^{-7}, 10^{-6}, and 10^{-5} M analog III, respectively (fig.4). β-ANP also exerted a similar inhibitory effect in the same system (not shown). The apparent IC_{50} values of analog III and β-ANP are 5.86×10^{-7} and 2.13×10^{-7} M, respectively.

The effect of analog III on the dose-response relationship of α-ANP was then investigated in terms of cGMP production in cultured VSMC. The results are shown in fig.5. The dose-response curve of α-ANP moves toward higher doses as the concentration of analog III is increased, while the maximum response of α-ANP appears to remain unchanged. From these results the ED_{50} values of α-ANP were estimated as 3.9×10^{-8} M in the absence of analog III and 1.4×10^{-7}, 4.5×10^{-7}, 8.9×10^{-7} and 3.5×10^{-6} M in the presence of 1×10^{-7}, 3×10^{-7}, 1×10^{-6} and 3×10^{-6} M analog III, respectively. The Schild plots [12] of these data are shown in fig.6, in which the $[ED_{50}/(ED_{50})_0 - 1]$ values are plotted vs concentration of analog III on a logarithmic scale, where $(ED_{50})_0$ denotes an ED_{50} in the absence of analog III. There exists a good correlation giving an equation of $y = 0.99x + 7.3$ and a correlation coefficient $r = 0.99$. From the intercept on the abscissa a pA_2 value of 7.45 was obtained for analog III as competitive antagonist.

3.5. *Effect of analog III on α-ANP-induced vasorelaxation*

Fig.7 shows the dose-response relationship of α-

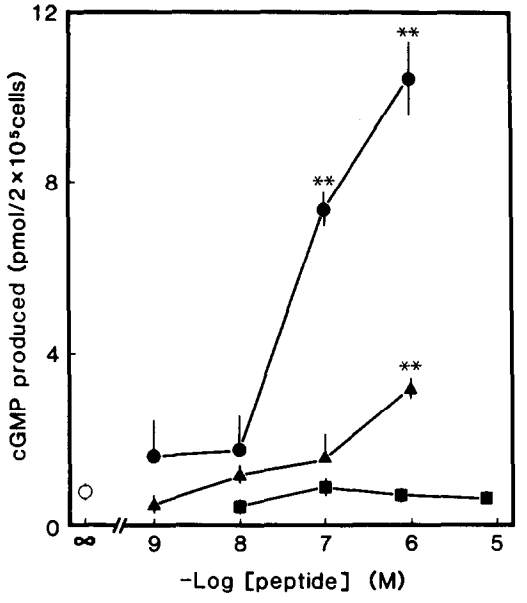


Fig.3. Effect of α-ANP (●), β-ANP (▲) and analog III (■) on intracellular cGMP production in rat VSMC. Results expressed as means ± SE ($N = 4$). Asterisks indicate significant difference vs control (* $p<0.05$, ** $p<0.01$).

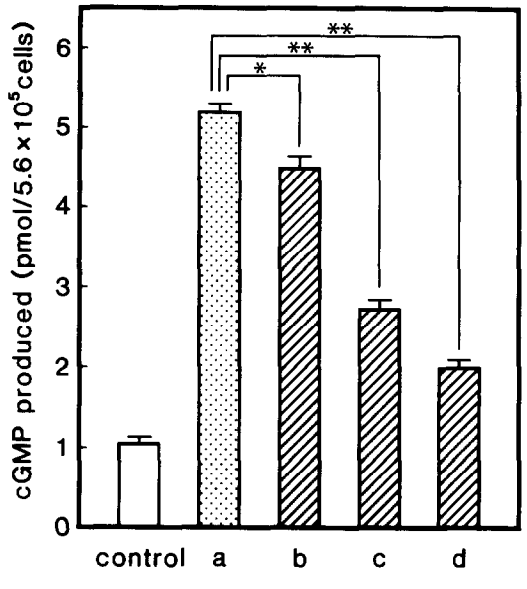


Fig.4. Inhibition of intracellular cGMP production by analog III in rat VSMC. Cells were incubated with 10^{-7} M α-ANP in the absence (a) or presence of 10^{-7} (b), 10^{-6} (c) or 10^{-5} M (d) analog III. Control contains no peptide. Results expressed as means ± SE ($N = 4$; * $p<0.05$, ** $p<0.01$).

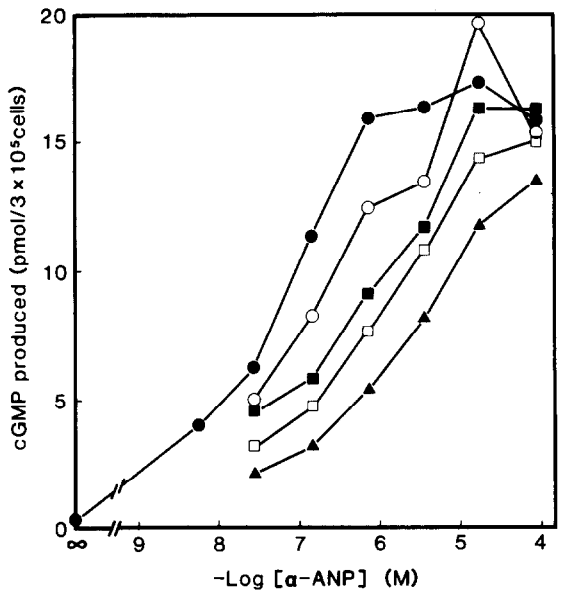


Fig.5. Effect of analog III on the dose-dependent cGMP production of α-ANP in rat VSMC. Cells were incubated with various concentrations of α-ANP in the absence (●) or presence of 10^{-7} (○), 3×10^{-7} (■), 10^{-6} (□), 3×10^{-6} M (▲) analog III. The experiment was peformed in duplicate. The maximim production of cGMP was estimated to be 16.3 pmol per 3×10^5 cells as an average value of those for four different concentrations of α-ANP from 7×10^{-7} to 9×10^{-5} M in the absence of analog III.

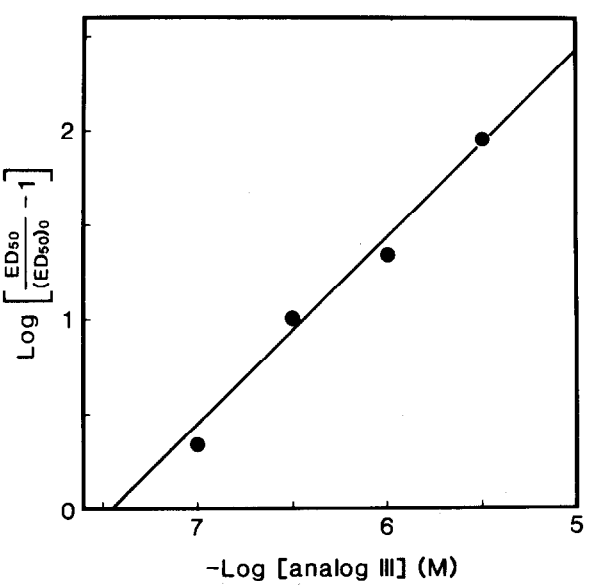


Fig.6. Schild plots for analog III in inhibition of α-ANP-induced cGMP production in rat VSMC. ED_{50} and $(ED_{50})_0$, concentrations of α-ANP inducing 50% of the maximum response in the presence and absence of analog III, respectively.

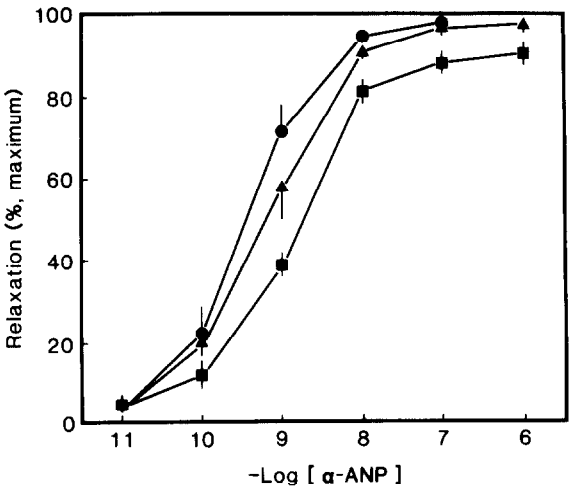


Fig.7. Effect of analog III on vasorelaxation induced by α-ANP. α-ANP was cumulatively added to the rat aortic strips partially contracted with PGF_{2a} in the absence (●) or presence of 10^{-7} (▲) or 10^{-6} M (■) analog III. Relaxations induced by 0.1 mM papaverine hydrochloride were taken as 100%. Results expressed as means ± SE ($N = 5$).

ANP in the relaxation of arterial strips previously contracted with $PGF_{2\alpha}$ in the presence or absence of analog III. The ED_{50} value of α-ANP was 4.6 (± 1.2) × 10^{-10} M when strips were contracted in the absence of analog III (control), and increased to 9.4 (± 4.0) × 10^{-10} and 19.2 (± 3.5) × 10^{-10} M (increase, $p < 0.01$, Tukey's test) in the presence of 1 × 10^{-7} and 1 × 10^{-6} M analog III, respectively. When tissues had previously been relaxed with 1 × 10^{-9} M α-ANP, however, no contraction occurred upon addition of 1 × 10^{-6} M analog III (not shown).

4. DISCUSSION

In a previous report, we synthesized β-ANP-(7–28) and found that the deletion of a pair of 6-residue N-terminal segments from β-ANP had no significant effect on biological properties of β-ANP, especially on its natriuresis [8]. Here, we have tried to synthesize a dicarba analogue [$Asu^{7,23}$]-β-ANP-(7–28), termed analogue III, in which one of the two S–S bonds of β-ANP-(7–28) is replaced by a CH_2–CH_2 linkage and one of the two N-terminal amino groups is deleted (fig.1). In contrast to β-ANP, which tends to be converted into α-ANP when incubated with human plasma [5] or cultured rat VSMC [6], analog III does not under-

go such conversion under the usual physiological conditions because of the presence of an Asu residue in the molecule.

We have found in a preliminary experiment that [Asu$^{7,23'}$]-β-ANP-(7–28) (analog III) is much less active (< 1%) than β-ANP in smooth muscle relaxation in vitro and behaves as a weak partial agonist in natriuresis in anesthetized rats (unpublished). On the other hand, Watanabe et al. [13] reported that a deamino-dicarba analog of α-ANP, [Asu^{7,23}]-α-ANP-(7–28), was less active (~ 10%) than α-ANP in vasorelaxation in vitro and natriuresis in rats, in which they found, however, that the maximum responses to this monomeric analog were the same as those to α-ANP when the concentration was sufficiently high. Therefore, the dimer seems to be affected more markedly by the substitution of an amino suberic acid for a cystine residue than the monomer. This cannot be explained as a sole effect of the substitution, but should be ascribed more to the fact that the substituted dimer can no longer be converted into the corresponding monomer. Thus, the dimer-to-monomer conversion seems to be of critical importance for β-ANP in manifestation of its biological activities.

It is generally accepted that cGMP mediates vascular smooth muscle relaxation evoked by α-ANP [14]. α-ANP has specific receptors on cultured rat VSMC and is known to stimulate intracellular guanylate cyclase [9]. In this respect we have been trying to measure cGMP production in VSMC for characterization of synthetic ANP analogs. The cultured cells used were found to be identical with those established by Hirata et al. [9] with respect to the dissociation constant and the maximum binding sites for α-ANP.

In the present investigation, we have found that the ability of analog III to displace ^{125}I-α-ANP specifically bound to VSMC is 1/10 that of α-ANP and 1/4 that of β-ANP (fig.2). In spite of this considerable binding to cells, analog III did not stimulate cGMP production in VSMC even at 10^{-5} M, while α-ANP exhibited remarkable stimulation with an ED_{50} value of 3×10^{-8} M (fig.3). These results suggest that analog III is either a competitive antagonist of α-ANP or a so-called clearance receptor (C-receptor) agonist, which is able to bind to the C-receptor but not to the bioactive receptor (B-receptor), only the B-receptor being coupled to guanylate cyclase [14,15]. We therefore examined analog III with special reference to its effect on the α-ANP-induced production of intracellular cGMP in VSMC. The results shown in figs 4 and 5 clearly indicate that analog III is a specific antagonist of α-ANP. The Schild analysis [12] of these data has revealed that the inhibitory profile of analog III is competitive and its pA_2 value is 7.45 (fig.6). Thus, it has become clear that analog III binds to the guanylate cyclase-coupled ANP receptor but fails to stimulate the enzyme, probably because of the lack of dimer-to-monomer conversion at the receptor site. This antagonism of analog III has also been observed in α-ANP-induced relaxation of rat aortic strips (fig.7). This observation supports the view that the signal transduction of α-ANP is mediated by cGMP in vasorelaxation. However, the antagonism was observed only when analog III was introduced at the stage of contraction with PGF_{2a} and prior to the addition of α-ANP. The tissues once dilated with α-ANP were not contracted by the addition of analog III. The precise mechanism of signal transduction of α-ANP in smooth muscle relaxation, therefore, remains to be elucidated.

Although the characteristic pattern of action of β-ANP in vasorelaxation and natriuresis [2,3] may be explained, at least in part, by the slow conversion of β-ANP into α-ANP in human plasma or at target tissues [5–7,16], it has been a matter of controversy as to whether β-ANP itself is biologically active. The present investigation has also shown that β-ANP inhibits the α-ANP-induced production of intracellular cGMP in cultured VSMC with an apparent IC_{50} value of 2.13×10^{-7} M, which is about one-third that of analog III and this ratio is comparable to that one between the IC_{50} values of β-ANP and analog III for receptor binding. This finding as well as those described above strongly suggests that β-ANP is inherently inactive, if not completely inactive, and can even behave as a competitive antagonist of α-ANP as long as it exists in the form of dimer. However, the detailed mechanism of vasorelaxation and natriuresis of β-ANP remains to be elucidated.

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