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The role of the C-terminal region of rat brain natriuretic peptide in receptor selectivity

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Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) have different C-terminal tail structures compared with the rather conservative ring structures which consist of 17 amino acid residues. To examine the different effects of the tail structures of ANP and BNP on their interaction with receptors, we synthesized several peptide analogs and measured their biological actions in three different assay systems. Deletion of the C-terminal tail from rat BNP did not effect the vasorelaxation activity against rat aorta, but it promoted cGMP production in cultured rat aortic smooth muscle cells (RASMC). Deletion of the C-terminal tail from rat ANP diminished both vasorelaxant and cGMP producing activities. In a binding competition assay with RASMC and [^{125}I]rat ANP-(1–28), the competition activities of both ANP and BNP were greatly reduced by C-terminal deletion. In addition, we obtained agonists with novel receptor selectivity.

Natriuretic peptide; C-terminal region; Receptor selectivity; Diuretic effect

1. INTRODUCTION

Natriuretic peptides (NPs) have natriuretic, diuretic, and hypotensive properties and are classified into three types, atrial natriuretic peptides (ANP), brain natriuretic peptides (BNP) and C-type natriuretic peptides (CNP) [1–7]. They have an intramolecular disulfide bond which forms a ring structure with 17 amino acid residues. Sequence homology in the ring region is higher than that in other regions, such as the N-terminal extension and C-terminal tail regions. The fact that CNP has no C-terminal tail structure suggests that the C-terminal tail regions of ANP and BNP may exert different effects through different receptor subtypes. Receptors for NPs are also classified into three types, type A receptor (GC-A), type B receptor (GC-B) and clearance receptor (C-receptor) [8–14]. GC-A and GC-B have intracellular guanylate cyclase catalytic domains to produce cGMP as a second messenger, and are therefore identified as biological receptors. The C-receptor exists as a dimer differently from GC-A and GC-B, and has been proposed to participate in the clearance of ANP [15]. The proportion of these receptors varies from one tissue to another [12,16–20], and the physiological roles of the respective receptors are still controversial. In this study we used three assay systems to evaluate the change in receptor-selectivity profiles of synthesized analogs. We report on compounds with novel receptor selectivity and the distinct biological effects of the C-terminal structures of rat BNP and rat ANP.

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC-A, ANP type A guanylate cyclase receptor; GC-B, ANP type B guanylate cyclase receptor; C-receptor, ANP clearance receptor; RASMC, rat aortic smooth muscle cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Rat ANP-(1–28), rat BNP-45 and rat CNP-22 were obtained from Peptide Institute, Inc. (Osaka, Japan). Other peptide analogs were prepared by solid phase synthesis, and purified by reverse-phase chromatography.

2.2. *Assays used*

Vasorelaxation was measured as in [21]. Rat aortic strips (2 mm × 5 mm, from male Sprague–Dawley rats) were mounted on stainless-steel hooks under 1 g resting tension. The strips were then equilibrated in a modified Locke–Ringer solution with 95% O_2 and 5% CO_2. After contraction of the strips with about 1 μM PGF2α, peptide solution was added cumulatively. cGMP producing activity in cultured rat aortic smooth muscle cells (RASMC) was measured as in [22]. Cells were washed twice with Hanks solution containing 20 mM HEPES (pH 7.4), 0.1% bovine serum albumin (Solution A) in the presence of 0.5 mM 1-methyl-3-isobutyl xanthine, then treated with the peptide dissolved in the same solution. After 10 min at 37°C, cells were homogenized in 6% trichloroacetic acid. The concentration of cGMP was determined using a radioimmunoassay kit from Yamasa (Tokyo). Binding competition assay was performed with RASMC as in [22]. RASMC cells were incubated with [^{125}I]rat ANP-(1–28) (Amersham, Tokyo) dissolved in Solution A in the presence or absence of non-labeled peptides for 4 h on ice. After washing with Solution A, cells were lysed with 1 M NaOH and counted for their radioactivity. Diu-

retic activities were measured with anesthetized Sprague–Dawley rats (male, 8–9 weeks old) as in [21]. Saline was insufed at 0.1 ml/min/kg through a femoral vein catheter and peptide solution (100 μl) was added intravenously. Urine was collected at 5-min intervals from the bladder through a polyethylene tube.

3. RESULTS

3.1. *Three assay systems to evaluate receptor selectivity*

Distribution of NP receptors varies in tissues and cultured cells. Suga et al. [19] reported that PC12 cells expressed mainly GC-A, while cultured RASMC expressed the C-receptor predominantly and a small prportion of GC-B as the biological receptor. GC-A and GC-B have the affinities of ANP≧BNP>>CNP and CNP>>ANP>BNP, respectively [23]. The vasorelaxant activities of NPs to rat aorta were ANP≧BNP>>CNP (Table I, Figs. 1A and 2A, and [19]). This indicates that GC-A is predominantly expressed as biological receptor in rat aorta. In other words, the vasorelaxant activity of a peptide corresponds to its response to GC-A. In a similar manner, the level of cGMP produced in cultured RASMC by a peptide corresponds to its response to GC-B, since GC- B is the predominant biological receptor in cultured RASMC. The abundant C-receptor present in cultured RASMC had no effect on cGMP production. This was confirmed by an experiment in which an excess amount of C-ANF$_{(4-23)}$, a C-receptor-specific ligand, co-existed during ligand treatment in the cGMP production assay. C-ANF$_{(4-23)}$ at 1 μM did not alter the dose-dependent profiles of any compounds tested (data not shown). We also used C-ANF$_{(4-23)}$ in a vasorelaxant assay to examine the effects of the co-existing C-receptor, but failed in our attempt because C-ANF$_{(4-23)}$ itself exhibited small but significant vasorelaxation potency at 1 μM (data not shown). Interaction of a peptide with C-receptor can be evaluated by binding competition assay with cultured RASMC and [^{125}I]rat ANP-(1–28).

3.2. *Effects of C-terminal structures on receptor selectivity*

For the molecular design of synthetic analogs, each molecule was divided into three regions, the N-terminal head, C-terminal tail and ring regions. To determine which region is most important in recognizing the respective receptors, we synthesized deletion analogs of rat NPs as well as chimeric analogs and compared their response to receptors using the three assay systems described above. The results are shown in Table I. Rat ANP-(7–23) (compound 2) was less active than rat ANP-(1–28) (compound 1) in all assay systems which is consistent with previous reports [24,25]. In contrast, rat BNP-(23–39) (compound 5), a ring analog of rat BNP-45, had a similar activity to rat BNP-45 (compound 3) in the vasorelaxation assay. The cGMP level induced by compound 5 was four times higher than that by rat BNP-45. Binding competition activity was greatly lowered. Rat CNP-(6–22) (compound 7), an N-terminal truncated analog of rat CNP-22, was comparable to rat CNP-22 (compound 6) in both vasorelaxation and cGMP producing activities, while binding competition activity was lower than that of rat CNP-22. Compounds 8 and 11 are chimeric analogs with BNP-type C-terminal tail regions. Compound 8, a chimeric analog which combines rat ANP-(1–28) and rat BNP-45, was eight times, two times and ten times less active than its original peptide, rat ANP-(1–28), in vasorelaxation, cGMP, and binding competition assays, respectively. Compound 11, a chimeric analog combining rat CNP-22 and rat BNP-45, was four times less active than the original rat CNP-22 peptide in both vasorelaxation and cGMP

Table I

Biological activities of rat natriuretic peptide analogs

Compounds		Vasorelaxation* EC_{50} ($\times 10^{-10}$ M)	cGMP production** (fmol/10^5 cells)	binding competition*** IC_{50} ($\times 10^{-9}$ M)
1	rat ANP-(1–28)	3.23 ± 0.52 [13]	227 ± 13 [8]	0.0622± 0.0083 [7]
2	rat ANP-(7–23)	29.2 ± 5.4 [4]^a	162 ± 16 [3]^a	5.22 ± 0.66 [8]^a
3	rat BNP-45	3.64 ± 0.99 [8]	139 ± 18 [10]	4.50 ± 0.57 [7]
4	rat BNP-(23–45)	2.62 ± 1.49 [4]	386 ± 22 [3]^b	7.34 ± 1.63 [3]
5	rat BNP-(23–39)	1.83 ± 0.52 [4]	553 ± 76 [5]^b	256 ± 131 [4]^b
6	rat CNP-22	48.9 ± 9.6 [6]	497 ± 24 [10]	17.1 ± 2.9 [4]
7	rat CNP-(6–22)	42.7 ± 9.4 [7]	504 ± 32 [3]	292 ± 31.4 [3]^c
8	rat ANP-(1–23)-rat BNP-(40–45)	26.5 ± 4.7 [3]^a	122 ± 2 [3]^a	0.624 ± 0.126 [4]
9	rat BNP-(14–39)-rat ANP-(24–28)	6.27 ± 1.99 [4]	966 ± 41 [3]^b	1.31 ± 0.24 [4]
10	rat CNP-22-rat ANP-(24–28)	21.3 ± 2.2 [10]	608 ± 22 [3]^d	3.46 ± 0.99 [4]
11	rat CNP-22-rat BNP-(40–45)	183 ± 33 [10]^c	89.6 ± 6.0 [3]^c	12.0 ± 2.18 [3]

Values are means ± S.E.M. with number of experiment shown in [ ]. *Vasorelaxation activity against rat aortic strips is expressed as EC_{50}, the concentration of the peptide which causes 50% of the maximal relaxation achieved with 0.1 mM papaverine hydrochloride. **The amount of cGMP in cultured RASMC induced by each peptide at 1 μM. ***Binding competition activity is expressed as IC_{50}, the concentration of the peptide which causes 50% inhibition of the binding of [^{125}I]rat ANP-(1–28) to cultured RASMC. Significant differences by Dunnett multiple comparison are as follows: $^aP<0.01$ vs. rat ANP-(1–28); $^bP<0.01$ vs. rat BNP-45; $^cP<0.01$ vs. rat CNP-22; $^dP<0.05$ vs. rat CNP-22.

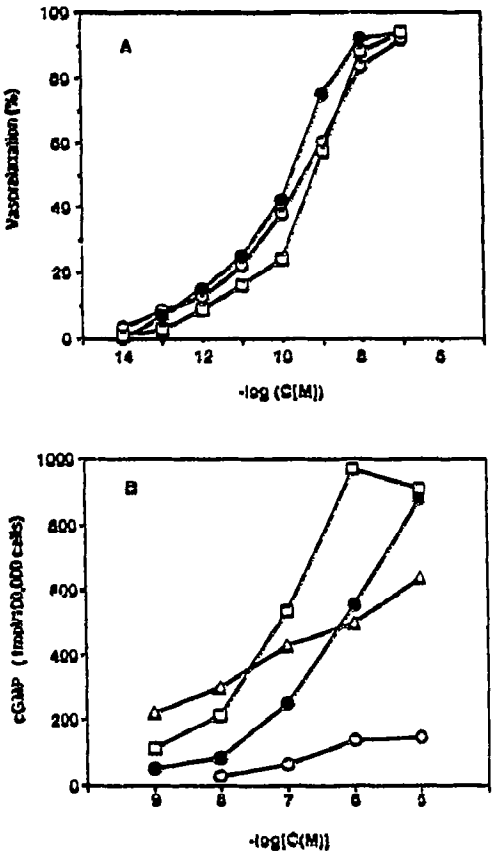

Fig. 1. Dose-dependent curves of biological receptor-selective compounds in vasorelaxation (A) and cGMP (B) assays. Procedures are described in detail in section 2. The relaxation activities were normalized with that of 0.1 mM papaverine hydrochloride as 100%. ▽, rat BNP-45; ●, rat BNP-(23–39); □, compound 9; △, rat CNP-22.

assays, but twice as active in the binding competition assay. Chimeric analogs with rat ANP-type C-terminal tail regions showed the quite distinct alteration in activities from those with BNP-type tails. With compound 9 and 10 that have ANP-type C-terminal tail regions, the situation was quite distinct. Compound 9, a chimeric analog combining rat BNP-45 and rat ANP-(1–28), had comparable vasorelaxant activity to rat BNP-45, while it had increased activities in cGMP and binding competition assays. Compound 10, a chimeric analog combining rat CNP-22 and rat ANP-(1–28), was more active than rat CNP-22 in all assays. The dose-dependent curves of several compounds in cGMP assay as well as vasorelaxation assay are shown in Figs. 1 and 2. Interestingly, compounds 9 and 10 showed the higher cGMP producing activities (at least ten times) than rat CNP-22. As shown in Fig. 1 and Table I, compound 7 showed a greater preference for the GC-B receptor than rat CNP-22 did due to great deterioration in the affinity for the C-receptor. Compounds 5 and 9 exhibited novel and interesting receptor selectivity (selectivity for so-called biological receptors). Among others, compound 5 was a very effective agonist of GC-A and GC-B receptors with extremely low affinity to the C-receptor.

3.3. *Diuretic effects of the ring analogs*

Diuretic activities of rat ANP-(7–23) and rat BNP-(23–39) are shown in Table II. Rat ANP-(7–23) exhibited very weak activity as expected from its low activity in all assays as described above. Although rat BNP-(23–39) was a very effective agonist of both GC-A and GC-B receptors, the diuretic activity of this compound was also lower than rat BNP-45, but the decrease in activity was not as drastic as rat ANP-(7–23).

4. DISCUSSION

It is known that the C-terminal tail region of rat ANP is important for its biological activity [24]. This is consistent with our results that rat ANP-(7–23) was less active than rat ANP-(1–28) in all assays tested. The results shown in Table I give us a rough idea about the ligand selectivities of the respective receptors. First, both GC-A and GC-B receptors seem to primarily require the ring structure and both have a preference for the BNP-type ring structure. GC-B also has a preference for CNP-type ring structure, suggesting that the amino acid residues that are common in the rings of BNP and CNP, but not in that of ANP, are important

Table II

Diuretic activities of rat ANP-(1–28) and BNP-45, and their ring analogs

Compound	Dose (nmol/kg)	Urine volume (μg/min)	Na excretion (μEg/min)	K excretion (μEg/min)
rat ANP-(1–28)	3	24.18±3.72	4.78±0.59	0.65±0.09
rat ANP-(7–23)	3	2.58±0.48[a]	0.37±0.16[a]	0.23±0.09[a]
rat BNP-45	3	27.33±4.04	5.48±1.01	1.17±0.17
rat BNP-(23–39)	3	8.08±1.01[b]	1.53±0.21[b]	0.25±0.04[b]

Values are expressed as mean±S.E.M. (n=6).
[a] P<0.01, significantly different from rat ANP-(1–28) by Turkey's test.
[b] P<0.01, significantly different from rat BNP-45 by Turkey's test.

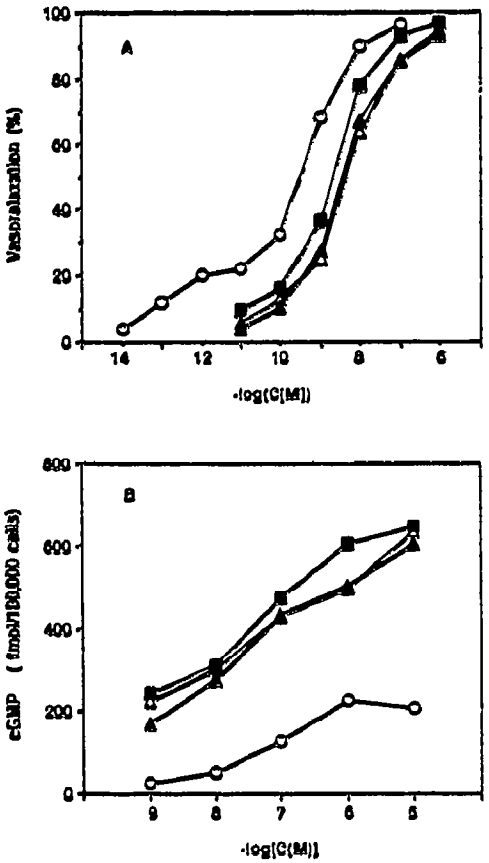

Fig. 2. Dose-dependent curves of compounds with high cGMP producing activities. The results of vasorelaxation and cGMP assays are shon in A and B, respectively. Values are expressed in the same manner as in Fig. 1. ○, rat ANP-(1–28); △, rat CNP-22; ▲, rat CNP-(6–22); ■, compound 10.

for the specific interaction between ligands and GC-B. Secondly, the ring structure of ANP has a rather low affinity for either GC-A or GC-B. Extension of the C-terminal of ANP, but not of BNP, greatly improves its affinity for GC-A. In general, it appears that the ANP-type C-terminal tail promotes and the BNP-type tail deteriorates the specific interaction of ligands with the GC-A or GC-B receptor, although the magnitude of alteration differs somewhat from one ligand–receptor combination to another. Thirdly, the C-receptor has a high selectivity for rat ANP-(1–28), recognizing not only the ring structure but also the C-terminal region. Both rat BNP-45 and rat CNP-22 have weaker affinities for the C-receptor than rat ANP-(1–28). In all cases, binding activities are reduced by deletion of the C-terminal region.

Concerning the renal actions of NPs, the diuretic activities in most cases, but not always, showed good correspondence to the vasorelaxation activities [25]. Therefore, the great reduction in diuretic activity of rat ANP-(7–23) was quite reasonable, since its activity in both the vasorelaxation and cGMP assays was diminished. On the contrary, the diuretic activity of rat BNP-(23–39) was not what we expected from its vasorelaxation and cGMP activities. Although rat BNP-(23–39) has comparable vasorelaxation potency to rat BNP-45 and rat ANP-(1–28) and higher potency in cGMP assay, its diuretic activity was significantly lower than that of rat BNP-45 and rat ANP-(1–28). There is a report on the existence of GC-A and GC-B in kidneys [12]. Sudoh et al. [6] reported that CNP had diuretic activity in anesthetized rats, but the potency was at least 80 times lower than that of ANP-(1–28). Recently, Stingo et al. [26] reported that CNP exhibited anti-diuretic effects in anesthetized dogs with a dose comparable to that used in our study (Table II), suggesting that the GC-B receptor in kidney acts repressively on the diuretic activity of NP. Our observations with rat BNP-(23–39) that GC-A acts stimulatively and GC-B acts, repressively on diuresis coincides with that of Stingo et al. There might be a possible involvement of other types of NP receptors in diuresis.

The localization of CNP, an intrinsic ligand to GC-B [23], seems restricted to the pituitary gland and brain, such as hypothalamus, thalamus, cerebellum and midbrain, and there are no or very small detectable amounts of CNP in other peripheral tissues including circulating blood [27]. As for the tissue distribution of GC-B, it is also localized mainly in brain but also in some other peripheral tissues such as lung, kidney, colon and adrenal medulla [12,18,28]. Our observation that rat BNP-(23–39) effectively agonized GC-B suggests that this form of BNP might represent an intrinsic ligand for peripheral GC-B receptor. Recent reports show the existence of endopeptidases other than enkephalinase that can cleave specific bonds in the ANP molecule and other peptide hormones [29–31]. Therefore, it is possible that the ring by itself or similar form of BNP might be found naturally.

We obtained compounds with novel receptor selectivity in the present study. Rat BNP-(23–39) and compound 9 have high affinity for GC-A and GC-B, and very low affinity for the C-receptor, and therefore, are agonists with selectivity for the biological receptors. Rat CNP-(6–22) and compound 10 have improved GC-B selectivity compared to the original rat CNP-22. These compounds may provide good biological tools to characterize the physiological roles of GC-A and GC-B in further research.

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