Molecular Mechanism of Action by Atrial Natriuretic Peptide in Rat Vascular Smooth Muscle Cells

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The mechanism by which atrial natriuretic peptide (ANP) acts on cells remains obscure. Using cultured vascular smooth muscle cells (VSMC) from rat aorta, we studied the structure-activity relationship of α -human(h) ANP and attempted to clarify its cellular mechanism of action. Binding studies using a variety of synthetic α -hANP⁷⁻²⁸ analogs with deletion and substitution of amino-acid residue(s) within the ring structure and deamino-dicarba analogs with replacement of the disulfide bond with an ethylene linkage, suggested that the original cyclic structure is a minimum requirement for biological activity and the disulfide bond is not essential for receptor binding. The present study using VSMC loaded with a fluorescent Ca^{2+} indicator quin-2 revealed that α -rat(r) ANP has no effect on increases in cytosolic free Ca^{2+} concentration stimulated by angiotensin (A) II or arginine-vasopressin (AVP). While both vasoconstrictive hormones rapidly stimulated phosphatidylinositol (PI) response in VSMC, pretreatment with rANP did not affect AII- or AVP-induced PI response. However, AII-stimulated phosphorylation level of 20 K-dalton (Da) myosin light chain (MLC), a regulatory contractile protein of VSMC, was attenuated by pretreatment with rANP. These data suggest that ANP may not act at site of either agonist-induced membrane PI hydrolysis or intracellular Ca^{2+} -signal system, but may partly be involved in phosphorylation/dephosphorylation of 20 K-Da MLC in cultured rat VSMC.

ATRIAL natriuretic peptide (ANP) is a novel cardiac hormone with potent natriuretic and vasodilatory activities, which may play a pivotal role in the regulation of extracellular fluid volume and blood pressure.¹ It is generally accepted that the action of polypeptide hormone is initiated by binding to its specific receptor in

the cell membrane, thereby generating transmembrane signal(s). Immediately after discovery of α -human(h) ANP by Kangawa and Matsuo,² we demonstrated that cultured vascular smooth muscle cells (VSMC) from rat aorta have abundant specific receptors for α -hANP functionally coupled to the guanylate cyclase system.³ However, its cellular mechanism of action remains unknown. Using cultured rat VSMC as a model for its target cell, we studied the structure-function relationship of α -hANP and attempted to clarify the molecular mechanism by which ANP acts on cells.

Key words:

Atrial natriuretic peptide
Structure-activity relationship
Cytosolic free Ca^{2+} concentration
Phosphatidylinositol response
Myosin light chain phosphorylation

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This study was supported in part by Research Grants from the Ministry of Health and Welfare (60A-3, 62A-1, 63C-1) and the Ministry of Education, Science and Culture (62570530), Japan

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Fig. 1. Competitive binding of ^{125}I - α -hANP to rat VSMC by synthetic α -hANP analogs. Confluent cells were incubated with ^{125}I - α -hANP at 24°C for 60 min in the absence and presence of α -hANP $^{7-28}$ analogs (upper panel) and [Asu 7,23]hANP $^{7-28}$ analogs (lower panel). Specific binding was 95% of total binding. Each point is the mean of duplicate dishes.

MATERIALS AND METHODS

Binding Study

Rat VSMCs were cultured and used as previously reported.³ α -hANP $^{7-28}$ analogs with deletion and substitution of amino-acid residue(s) within the ring structure (7–23) and deaminodicarba analogs ([Asu 7,23] α -hANP $^{7-28}$) with replacement of the disulfide bond with an ethylene linkage were synthesized as described elsewhere.⁴ Binding study was performed essentially in the same manner as reported.⁵ In brief, confluent cells were incubated at 24°C for 60 min with 10^{-10} M ^{125}I -labeled- α -hANP $^{1-28}$ (Peptide Institute) in Hanks' balanced salt solution, pH 7.4, containing 0.1% bovine serum albumin in the absence and presence of α -hANP analogs. For measurement of guanylate cyclase activity, cells were incubated at 37°C for 10 min

with or without α -hANP analogs in medium containing 0.5 mM methylisobutylxanthine (Calbiochem-Behring) and intracellular cGMP content was determined by RIA.⁵

Measurement of Intracellular Free Ca^{2+} Concentration ($[\text{Ca}^{2+}]_i$)

For measurement of $[\text{Ca}^{2+}]_i$ in VSMC, dispersed cells were loaded with 50 μM quin-2 acetoxymethyl ester (Dojin) at 37°C for 20 min, washed and suspended at a concentration of $\sim 10^6$ cells/ml. Fluorescence was measured in a spectrofluorimeter with excitation of 339 nm and emission of 492 nm according to the method of Tsien et al.⁶

Phosphatidylinositol (PI) Response

For assessment of PI response, confluent cells were incubated with 25 μCi [^3H] myoinositol at

Fig. 2. Changes of $[Ca^{2+}]_i$ of rat VSMC in response to vasoactive hormones. Quin-2-loaded cells were challenged with 10^{-7} M angiotensin II (AII) and 10^{-6} M arginine-vasopressin (AVP) without (left panel) and with pretreatment of 10^{-6} M rANP (right panel).

37°C for at least 48 hrs. After washing, cells were incubated with angiotensin II (AII: Peptide Institute) or arginine-vasopressin (AVP: Sigma) at 37°C for 30 sec in the presence of 10 mM LiCl. Incubation was terminated by rapid aspiration of the medium and addition of 15% trichloroacetic acid (TCA). After washing TCA extract with diethylether, the samples were applied to 2 ml Dowex - 1×8 anion-exchange (Dow) column. The phosphate esters were eluted sequentially by eluants containing increasing concentrations of formate as described by Berridge et al⁷: inositol 1-phosphate (IP) with 0.1 M formic acid/0.2 M ammonium formate, inositol 1, 4-bisphosphate (IP₂) with 0.1 M formic acid/0.4 M ammonium formate and inositol 1, 4, 5-trisphosphate (IP₃) with 0.1 M formic acid/1 M ammonium formate.

Radioimmunoblotting of Myosin Light Chain (MLC)

To determine phosphorylation levels of 20 K-dalton (Da) MLC in VSMC, confluent cells were incubated at 24°C for one min with AII or AII plus rANP. Incubation was terminated by rapid aspiration of medium and addition of 20% TCA. TCA-insoluble proteins were subjected to glycerol-urea polyacrylamide gel electrophoresis to separate phosphorylated from unphosphorylated MLC as described by Perrier and Perry⁸. Radioimmunoblotting of 20 K-Da MLC was performed by the method of Hathaway and Haeberle⁹. After electroblotting, the nitrocellulose was first incubated with rabbit anti-

gizzard MLC serum, then with ^{125}I -labeled-protein A, and subjected to autoradiography. Phosphorylation levels of 20 K-Da MLC were measured with a laser densitometer to estimate the ratio of phosphorylated band (P) to unphosphorylated and phosphorylated bands (UP + P).

RESULTS

Both $[\text{Nle}^{12}]$ and $[\text{D-Ala}^9]$ hANP analogs were as potent as $\alpha\text{-hANP}^{7-28}$ (K_i : $2 - 3 \times 10^{-10}$ M) but $[\text{Des-Gly}^9]$ hANP was less potent (K_i : 10^{-9} M), whereas both $[\text{D-Asp}^{13}]$ and $[\text{Des-Ala}^{17}, \text{Gly}^{18}, \text{Ser}^{19}, \text{Gly}^{20}, \text{Leu}^{21}]$ hANP analogs were ineffective (Fig. 1; upper panel). In contrast, $[\text{Ile}^{12}]$ Asu analog was fully potent (K_i : 5×10^{-10} M) but both $[\text{Nle}^{12}]$ and $[\text{Met}^{12}]$ Asu analogs were less potent (K_i : $3 - 4 \times 10^{-9}$ M) than $\alpha\text{-hANP}^{7-28}$ (Fig. 1; lower panel). The stimulatory effects on cGMP generation by these analogs generally correlate well with their in vitro vasorelaxant activities of precontracted rat aorta⁴ but appear to be several orders of magnitude less than their binding activity (data not shown).

AII and AVP, both potent vasoconstrictive hormones, induced a rapid and transient increase in $[Ca^{2+}]_i$, followed by a sustained and plateau increase (Fig. 2; left panel). Since removal of extracellular Ca^{2+} by EGTA abolished the plateau phases, but not the transient phases of $[Ca^{2+}]_i$ increase by AII and AVP, the plateau phase is dependent on extracellular Ca^{2+} pool and the transient phase is most likely mobilized from

Fig.3. Effect of rANP on angiotensin II-induced phosphatidylinositol response in rat VSMC.

Cells prelabeled with [^3H] myoinositol were stimulated with various doses of angiotensin II at 37°C for 30 sec without ($\circ$) and with ($\bullet$) pretreatment of 10^{-7} M rANP. Water-soluble phosphoinositides metabolites (IP , IP_2 , IP_3) were extracted and separated by anion-exchange chromatography as described in Method.

Fig.4. Effect of rANP on angiotensin II (AII)-stimulated phosphorylation of 20 K-Da myosin light-chain (MLC) in rat VSMC.

Cells were incubated at 24°C for one min in the absence ($\square$) and presence of AII ($\square$) or AII plus rANP ($\boxtimes$). Phosphorylation levels of 20 K-Da MLC were determined by radioimmunoblotting method using MLC antibody. The ratio of phosphorylated band to unphosphorylated and phosphorylated bands ($\text{P}/\text{UP} + \text{P}$) of 20 K-Da MLC is shown. Statistically significant difference between each group is indicated.

intracellular Ca^{2+} pool. Prior addition of α -rat(r) ANP $^{1-28}$, however, affected neither the transient nor plateau phases of AII- or AVP-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 2; right panel). Both AII and AVP rapidly (within one min) stimulated

hydrolysis of the membrane phosphoinositides, generating IP , IP_2 and IP_3 in a dose-dependent manner (Fig. 3). However, pretreatment with α -rANP had no effect on PI hydrolysis stimulated by either AII (Fig. 3) or AVP (data not shown).

While AII rapidly (within one min) and remarkably ($p < 0.001$) phosphorylated 20 K-Da MLC, pretreatment with α -rANP induced partial, but significant ($p < 0.05$) decrease of AII-induced phosphorylation level of 20 K-Da MLC (Fig. 4).

DISCUSSION

The present binding study reveals that deletion of Gly 9 or several residues (17–21) and substitution of Asp 13 for D-isomer of synthetic α -hANP analogs lead to a partial or complete loss of receptor binding, while binding affinity was unaffected by replacement of Gly 9 or Met 12 with D-Ala and Nle, respectively. Furthermore, Asu analogs with replacement of the disulfide bond with an ethylene linkage retain binding activity. However, the potency of these analogs for stimulating guanylate cyclase was far less than their binding activity. These results are consistent with those of a recent report 10 in which there is no correlation between receptor binding affinity of ANP analogs and their potency for stimulating guanylate cyclase, suggesting the existence of multiple ANP receptors or binding sites. Our data also suggest that the original cyclic structure, composed of 17-amino-acid residues, is a minimum requirement for biological activity and the disulfide bond is not essential, but important for maintaining active

conformation.

Since $[Ca^{2+}]_i$ in VSMC plays a key role in the regulation of vascular smooth muscle contraction, the measurement of $[Ca^{2+}]_i$ using fluorescent Ca^{2+} indicators, such as quin-2 and fura-2, provides a valuable tool for understanding the mechanism of intracellular Ca^{2+} -signal system. Using quin-2-loaded cells, the present study clearly shows that vasoconstrictive hormones, such as AII and AVP, increase $[Ca^{2+}]_i$, whereas ANP does not inhibit AII- or AVP-induced rise of $[Ca^{2+}]_i$ in suspended VSMC. Our data are consistent with those by Capponi et al¹¹ in which ANP fails to affect AII-induced increase of $[Ca^{2+}]_i$ not only in quin-2-loaded rat VSMC, but also in bovine adrenal glomerulosa cells and rat renal cortical cells. Recent study,¹² however, has suggested that ANP decreases basal and AII-induced increase of the plateau phase, but not the transient phase of $[Ca^{2+}]_i$ in monolayer culture of fura-2-loaded rat VSMC. The reason for the discrepant results is not clear, but it may be partly accounted for by different cell systems (suspended vs monolayer) or different Ca^{2+} indicators (quin-2 vs fura-2) used.

Since ANP has been reported to block $^{45}Ca^{2+}$ efflux by norepinephrine even in the absence of extracellular Ca^{2+} ,^{13,14} it is suggested that ANP may inhibit Ca^{2+} release from intracellular store sites, possibly sarcoplasmic reticulum (SR). Furthermore, vasoconstrictive hormones, such as norepinephrine, AII and AVP, stimulates hydrolysis of PIP_2 to generate IP_3 , a putative second messenger for mobilizing Ca^{2+} from SR.¹⁵ Therefore, it is possible that the inhibitory effect of ANP on Ca^{2+} release from intracellular pool may be mediated by blocking agonist-induced PI response, thereby leading to inhibition of IP_3 production. In fact, it has recently been shown that ANP blocks norepinephrine-induced PI hydrolysis in rat aorta¹⁶ as well as in rabbit renal artery.¹⁷ On the contrary, the present study clearly demonstrates that ANP does not affect PI response stimulated by either AII or AVP. Several possibilities may explain the discrepant findings. First, ANP may more preferentially act to inhibit phospholipase (PL) C coupled to α_1 -adrenergic receptor than PLC coupled receptors for AII and AVP. Second, cultured VSMC may have functionally lost ANP-mediated feedback pathway to phosphoinositides metabolism during in vitro culture system. Finally, ANP may act at site(s) beyond PI response and intracellular Ca^{2+} -signal system to exert its effect(s).

It has been shown that phosphorylation of 20 K-Da MLC by Ca^{2+} -calmodulin-dependent MLC kinase is an important regulatory mechanism of vascular smooth muscle contraction.¹⁸ Using radioimmunoblotting method, the present study shows that ANP attenuates AII-induced phosphorylation level of 20 K-Da MLC, suggesting the possible involvement of ANP in the mechanism of phosphorylation/dephosphorylation of the contractile protein(s) in VSMC. In this regard, it is noteworthy that ANP inhibits phosphorylation of a specific protein with the apparent molecular weight of 17.6 K-Da stimulated by AII in bovine adrenal glomerulosa cells.¹⁹ Taken together, one might postulate that ANP may exert its diverse effects by inhibiting protein kinase(s) to decrease phosphorylation and/or stimulating phosphatase(s) to increase dephosphorylation of specific proteins for cellular responses.

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