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STRUCTURE-ACTIVITY RELATIONSHIPS OF ATRIAL NATRIURETIC FACTOR (ANF) III CORRELATION OF RECEPTOR AFFINITY WITH RELATIVE POTENCY ON ALDOSTERONE PRODUCTION IN ZONA GLOMERULOSA CELLS

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The activity of various fragments of ANF as inhibitors of aldosterone secretion and as competitors of [^{125}I] ANF (Arg^{101}-Tyr^{126}) binding to specific receptors was studied in bovine zona glomerulosa. Shortening or lengthening the N-terminal segment of ANF does not alter its biological activity while minimally altering affinity for its receptor. Removal of the C-terminal to Cys^{121} or expansion up to Arg^{128} leads to 1000-fold decrease in receptor affinity and activity. The results indicate the importance of the C-terminal segment of ANF in determining its active conformation.

Atrial natriuretic factor (ANF) appears to be synthethized in the form of a pre-propeptide with 152 residues consisting of a signal peptide and a propeptide (1,2). Initial studies on the purification of biologically active forms of ANF indicated that C-terminal fragments of length varying from 21 to 38 residues and including the essential Cys^{105}-Cys^{121} disulfide bridge displayed full diuretic, natriuretic and vasorelaxant activity (1,2). Larger forms of ANF containing the C-terminal active portion and extending further toward the N-terminal end were also active suggesting that the C-terminal portion might represent the natural form of ANF following proteolytic clevage. However no detailed structure-activity relationship of the shorter mature forms of ANF has been reported.

We have previously reported that ANF (Arg^{101}-Tyr^{126}) potently inhibits hormone-stimulated production of aldosterone in cultured bovine adrenal

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(12). Such a synergistic effect of these agents has been found in the secretion of hormones (2, 3, 4, 5, 7) as well as histamine release from mast cells (13) and lysosomal enzyme release from neutrophils (14).

Recently, deBustros et al. reported that TPA increases calcitonin (CT) secretion and its production at the transcriptional level from human medullary thyroid carcinoma cells (15). However, it is not yet determined whether the effect of TPA on CT secretion is mediated via C-kinase activation. Furthermore, the interaction of C-kinase with Ca^{2+} mobilization or with cAMP-dependent protein kinase (A-kinase) should be clarified.

In the present study, the effect of phorbol esters or synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG), in combination with A23187 or dibutyryl cyclic AMP (DBcAMP), was investigated in rMTC 6-23 cells, originated from rat transplantable medullary thyroid carcinoma (16, 17).

Materials and Methods

Materials; TPA, 4α-and 4β-phorbol 12,13-didecanoate, and A23187 were purchased from Sigma Chemicals Co. (St. Louis, MO). OAG from Avanti Polar-Lipids, INC. (Birmingham, AL). DBcAMP from Toyo Jozo Co. (Choshi, Japan).

Cell culture; rMTC 6-23 cells (American Type Culture Collection) were grown as monolayers in Dulbecco's modified essential medium (Flow) supplemented with 15% horse serum (Gibco) in a humidified atmosphere with 5% CO_2 95% air.

Secretion experiment; After preincubation with serum-free Ham's F12 medium (Flow) for 15 min, confluent cells on replicate dishes were incubated with the medium containing test agents or vehicle alone for 60 min except for the time course study, as described previously (18). Then, the medium was collected, centrifuged, and kept at -20C until assayed. CT was measured by radioimmunoassay using human CT antibody (19), which completely cross-reacted with rat CT. CT concentration was corrected by cell protein measured by the method of Lowry et al.(20).

The results were expressed as the mean $\pm$ SEM. Statistical significance was assessed by Student's t-test.

Results

The time course effect of TPA and/or A23187 on CT secretion is shown in Fig.1. The stimulatory effect of TPA was sustained as compared to that of A23187. The effect of TPA was markedly enhanced at 60 and 90 min, when combined with A23187. At 60 min CT release induced by 250 nM A23187 was 130% of control, and that induced by 32 nM TPA was 160% of control, whereas the value

three days in 24-well plates (10^6 cells/well) in F-12 medium supplemented with 10% horse serum, 2% fetal calf serum and antibiotics (3). Cells were stimulated for three hours without serum and containing 0.2% lysosyme, 1 uM PGE_1 and the indicated concentration of peptides. All peptides were dissolved in 0.1 M acetic acid and concentrated stock solutions were diluted in final culture medium immediately prior to incubation. Aldosterone production was measured in the medium by radioimmunoassay using [^{3}H] aldosterone and anti-aldosterone antibody provided by Alain Bélanger (Québec City, Québec).

Radio-receptor assay

[^{125}I] ANF (101-126) was prepared by iodination with chloramine T and mono-iodinated peptide (500 Ci/mmol) was purified by reverse-phase HPLC and immuno-affinity chromatography (4). [^{125}I] ANF was incubated for 90 min at 25^{o}C in 1 ml of buffer containing 50 mM Tris-HCl pH 7.4, 5 mM $MnCl_2$, 0.1 mM EDTA, 0.5% heat-inactivated BSA, 30 µg of adrenal zona glomerulosa membrane protein and various concentrations of peptide fragments (4). Membrane-bound [^{125}I] ANF was separated by filtration and washing on GF/C glass fiber filters.

Data analysis

All dose-response curves were analyzed by non-linear regression using a four parameter logistic equation providing estimates for the asymptotic maximal and minimal responses, the slope factor and the IC_{50} of the curve (10). For radio-receptor assay, the inhibitory constant K_i was approximated according to Cheng and Prusoff (11). Bioassay IC_{50} and receptor assay K_i are reported as pD_2 (i.e. - log IC_{50}) and pK_i (i.e. - log K_i), respectively.

RESULTS

Removal of both Arg^{101} and Arg^{102} leads to a 10-fold reduction in affinity for ANF receptors (fig. 1) without significant loss of biological

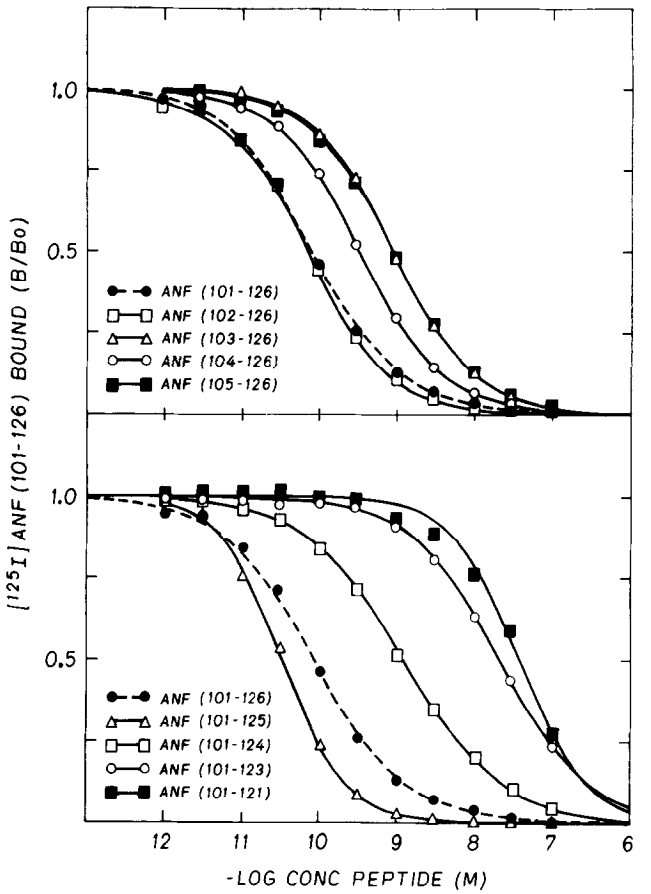

Fig. 1 Competition curves of [^{125}I] ANF (101-126) binding to zona glomerulosa membrane receptor in the presence of fragments missing residues at the N-terminal End (upper panel) or the C-terminal end (lower panel).

potency (fig. 2). Further exclusion of Ser^{103} and Ser^{104} is without effect in either system. The absence of Tyr^{126} does not reduce the affinity or the activity of the peptide. However, removal of Arg^{125} and Phe^{124} leads to a 100-fold reduction in both receptor affinity (fig. 1) and biological potency (fig. 2) of ANF. Suppression of Ser^{103} and Arg^{102} does not reduce the activity of the peptide any further. Simultaneous removal of residues at both N-terminal and C-terminal ends leads to additive effects on receptor affinity and biological activity (Table I). Extension of the N-terminal end up to Glu^{54} only slightly reduces both receptor affinity and biological

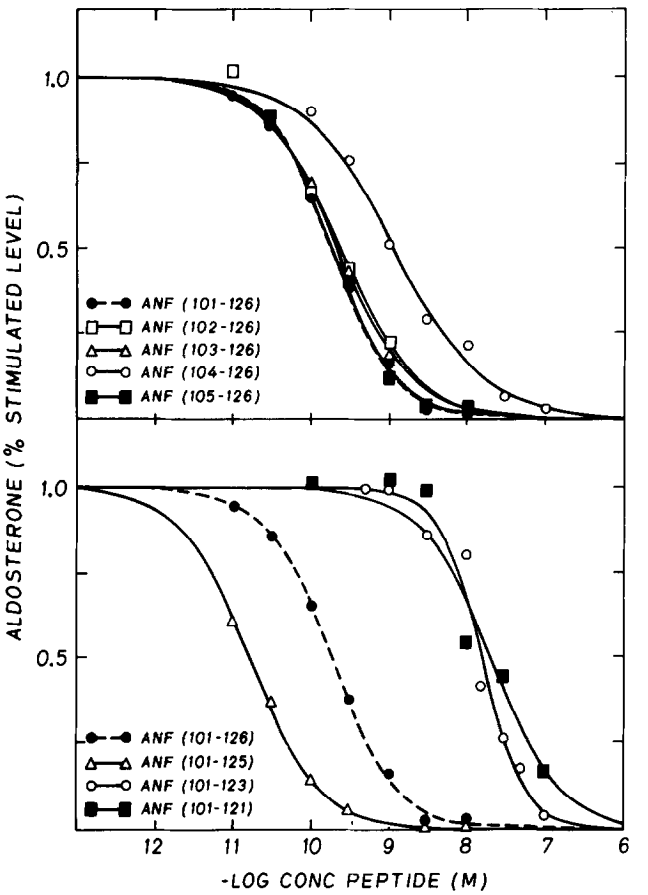

Fig. 2 Dose-response curves for the inhibition of PGE_1-stimulated secretion of aldosterone by ANF fragments missing residues at the N-terminal end (upper panel) or the C-terminal end (lower panel).

activity. In contrast, addition of Arg^{127} and Arg^{128} profoundly reduces receptor affinity and biological activity at least by 1000-fold (Table I). Substitution of methionine for Ile^{110} in the loop is the only difference with human ANF (12). Such a substitution does not alter the receptor affinity or the biological activity as compared with rat ANF (Table I).

There is a high correlation ($r = 0.945$) between the affinity for receptor and the biological activity as inhibitor of aldosterone production in vitro. All active fragments behave as full agonists and inhibite by 76% PGE-stimulated secretion of aldosterone (data not shown).

TABLE I

Correlation of biological potency with receptor affinity of ANF fragments[d]

	Receptor affinity (pK_i)[a]	Biological activity (pD_2)[b]
ANF (101-126)	10.3 ± 0.1	9.7 ± 0.05
ANF (102-126)	10.3 ± 0.1	9.7 ± 0.2
ANF (103-126)	9.3 ± 0.1	9.7 ± 0.2
ANF (104-126)	9.4 ± 0.1	9.2 ± 0.8
ANF (105-126)	9.2 ± 0.01	9.2 ± 0.8
ANF (101-125)	10.7 ± 0.1	10.7 ± 0.3
ANF (101-124)	9.1[c]	---
ANF (101-123)	7.8[c]	8.2 ± 1
ANF (101-121)	7.2 ± 0.02	7.7[c]
ANF (103-125)	9.2 ± 0.1	9.5 ± 0.2
ANF (103-123)	6.6 ± 0.2	7.2[c]
ANF (96-126)	10.3 ± 0.1	10.1 ± 0.06
ANF (54-126)	9.8 ± 0.02	9.5 ± 0.5
hANF[e]	10.1 ± 0.1	9.8 ± 0.2
ANF (94-128)	7.2	6

[a] -log K_i ± S.E.M. of duplicate receptor binding assay experiments with [^{125}I] ANF(101-126).

[b] -log IC_{50} ± S.E.M. of duplicate bioassay experiments for inhibition of PGE_1-stimulated aldosterone secretion.

[c] single experiment

[d] colleration r = 0.945 (n = 13, p < 0.01)

[e] human ANF

DISCUSSION

The adrenal zona glomerulosa system provides a simple, sensitive and reliable model for studying structure-activity relationships of ANF. The results obtained show that residues on the terminal side of the essential disulfide bridge are not part of the pharmacophore, although the Arg^{101} and Arg^{102} appear to stabilize peptide-receptor interaction and increase

receptor affinity (fig. 1, Table I). In contrast, all residues on the C-terminal end of the disulfide bridge are important, except perhaps Tyr^{126} which, besides its use for radio-iodination, does not contribute to receptor affinity or biological activity. Interestingly the presence of the extraneous Arg^{127} and Arg^{128} appears to drastically interfere with peptide-receptor interactions. The distinct location of the encoding region for these two residues in the rat gene (12) and their absence in all forms of ANF purified so far suggests that these two residues might be cleaved soon after nascent prepro-ANF is synthesized. However, the low relative potency of synthetic ANF (94-128) suggests that forms of ANF extending up to Arg^{128} would not be detected in any bioassay during purification procedures. The equipotency of rat and human ANF in bovine receptor assay and bioassay confirms the high degree of homology of this factor among mammalian species. It also indicates that residue^{110} in the cycle is not crucial for activity. Those results are in very good agreement with parallel studies performed with the same ANF fragments in *in vivo* rat natriuresis (6) and *in vitro* on chick rectum (6) and rabbit aorta relaxation (7). The high degree of agreement among these diverse systems suggests that vascular and non vascular ANF receptors might be pharmacologically similar. So far none of the fragments behaved as a partial agonist or as an antagonist. Testing of synthetic analogs of ANF will provide further characterization of the structure-activity relationship of atrial natriuretic factor and the requirements of its receptor.

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