ANANTIN — A PEPTIDE ANTAGONIST OF THE ATRIAL NATRIURETIC FACTOR (ANF)

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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(Received for publication August 18, 1990)

Anantin, a peptide binding to the receptor of the atrial natriuretic factor (ANF) was isolated from a strain of *Streptomyces coerulescens*. The molecule consists of 17 natural L-amino acids which form a peptidic ring system. It has a MW of 1,871.0. The chemical composition is $C_{90}H_{111}N_{21}O_{24}$.

The compound was found to bind competitively to ANF-receptors from bovine adrenal cortex ($K_d = 0.61\ \mu M$). Furthermore, it dose-dependently inhibited the ANF-induced intracellular cyclic guanosine monophosphate accumulation in bovine aorta smooth muscle cells. At the same concentration no agonistic effects were detectable in these cells. Thus, anantin is considered to be the first microbially produced antagonist of the cardiac hormone, ANF.

Atrial natriuretic factor (ANF) is synthesized as a pre-pro-hormone in the mammalian atrium and is stored as the pro-hormone ANF (1-126) in granules[1]. ANF is released into plasma following atrial stretch, predominantly in the form ANF (99-126)[2,3].

ANF binds with high affinity to membrane receptors[4]. Interaction with such functional receptors results in increased activity of the particulate guanylate cyclase and thus in accumulation of cyclic guanosine monophosphate (cGMP) in tissues and cells[5]. This has suggested that cGMP is the mediator of the physiological effects of ANF[6]. The main target of ANF is the kidney, where it enhances the glomerular filtration rate, renal blood flow, urine volume and the excretion of sodium. In addition, ANF is also a very potent direct vasodilator of the peripheral blood vessels[7]. In general terms, ANF apparently is actively involved in the blood pressure homeostasis. However, the exact (patho)physiological role of ANF has not yet been evaluated, mainly due to the lack of an antagonist†.

To screen for compounds binding to the ANF-receptor, a binding assay was used based on the well characterized bovine adrenal cortex membranes[9,10]. Binding activities were functionally characterized by their effect on intracellular cGMP in bovine aorta smooth muscle cells. These cells show dramatically increased intracellular cGMP levels upon stimulation with ANF[11] and are therefore suitable to distinguish between antagonists and agonists of ANF.

Here we describe the discovery and characterization of anantin, the first known ANF-antagonist of microbial origin, and of des-phe-anantin, a weakly active side compound which has been detected in the course of the isolation work.

† In the course of our studies an ANF-antagonist synthesized by chemical modification of ANF was published[8].

Materials and Methods

Production Organism

The production organism was isolated from a soil sample taken near Salt Lake City, Utah, U.S.A. After isolation, the culture segregated into two different morphovariants, a blue-green type, which was assigned the DSM No. 4777 and a white one, DSM 4778. An identification carried out by DSM (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) according to WILLIAMS *et al.*[12] showed that the blue-green and the white variant both belonged to the species *Streptomyces coerulescens*. The white type was considered to be a nonsporulating variant of the green one. Both of them were found to produce anantin.

Fermentation Process

S. coerulescens was grown and maintained on an agar medium consisting of malt extract (Difco) 10 g, glucose 4 g, yeast extract (Difco) 4 g, agar (Difco) 20 g in 1-liter tap water; pH was adjusted to 7.0 before sterilization. The cultures were grown at 30°C for 7 days.

For all liquid cultures, a medium was used consisting of soy meal 2%, D-mannitol 2% and a defoamer, polypropyleneglycol 2020 0.1%; pH was adjusted to 7.4 before sterilization. Inocula were grown in 500-ml shake flasks containing 100 ml medium. The flasks were inoculated with spores or aerial mycelium and incubated at 30°C for 48 hours on a rotary shaker. The contents of ten flasks were transferred into a 10-liter fermenter, stirred at 30°C with 360 rpm at an aeration rate of 0.4 v/v/m for 24 hours, before being inoculated into a fermenter containing 200 liters medium. This production fermentation was run at 30°C, with an aeration rate of 0.3 v/v/m and a stirrer speed of 600 rpm for 96 hours. The production of anantin was followed either by the binding assay or by an HPLC assay.

The Screening Model

Various organisms isolated from soil were cultured in shake flasks (500-ml Erlenmeyer flasks containing 100 ml of the medium indicated above) at 30°C for 5 days on a rotary shaker. 10 ml samples of the culture broth were centrifuged and the supernatant applied to Sep-Pak^R RP_{18} columns. After washing with water, the column was eluted with methanol and the eluate dried *in vacuo* and then redissolved in 0.5 ml of DMSO. 5 μl of this solution was tested in the ANF-receptor assay. For the functional characterization of the binding activities the effects on intracellular cGMP in bovine aorta smooth muscle cells were analyzed.

ANF-Receptor Assay

Membranes from bovine adrenal cortex were prepared as described[9]. Frozen aliquots containing 1 g of original cortex tissue (6 mg protein) per 1 ml of 75 mM Tris-HCl, pH 7.4, 25 mM $MgCl_2$, 1 mM dithiothreitol and 250 mM sucrose were used. Membrane aliquots were thawed and centrifuged at 39,000 × *g* for 15 minutes, the pellet resuspended in 10 ml of 50 mM Tris-HCl, pH 7.6, 500 mM $MgCl_2$, 1 mM EDTA and 0.5% bovine serum albumin (buffer A). The incubation samples were prepared by mixing (1) 100 μl membrane suspension containing 60 μg protein, (2) 50 μl ^{125}I-rat ANF (99-126) in buffer A (25,000 cpm or 20 pM final concentration), (3) 95 μl buffer A, and (4) 5 μl of DMSO containing fermentation broth extracts, dilutions thereof or samples from the downstream processing. The mixtures were incubated for 2 hours at 20°C. Bound and free radioligand were then separated by rapid filtration using Whatman GF/C glass fiber filters presoaked with buffer A. After washing the filters three times with 250 μl of buffer A, the filters were cut and the radioactivity measured in a γ-counter. The non-specific binding was defined as the binding in the presence of 10^{-7} M rat ANF (99-126) and was found to be 10% of the maximum binding (3,000 cpm).

cGMP Assay

Bovine aorta smooth muscle cells were obtained by enzymatic treatment of aorta with collagenase and elastase. The cells were grown in DULBECCO's modified Eagle medium (DMEM) containing 10 mM HEPES, pH 7.3, 10% fetal calf serum, 100 U/ml benzylpenicillin and 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM glutamine, 1 mM sodium pyruvate[9]. They were subcultured in the same medium after detachment by trypsinization and used between passage 13 and 15.

For the cGMP assay, 1×10^5 confluent cells in 24-well Costar culture dishes were maintained at 37°C in DMEM containing 25 mM HEPES, pH 7.3, and 2% bovine serum albumin. The ANF-ligands were

then incubated for 5 minutes at 37°C after a 5-minute preincubation with 0.5 mM of the phosphodiesterase-inhibitor isobutylmethylxanthine (IBMX). The medium was carefully aspirated and 0.75 ml of cold 6% TCA was added to the plates to lyse the cells. The cellular extracts were centrifuged for 20 minutes at 2,000 × *g* and the supernatants extracted three times with 5 ml of ether. The water-phase was lyophilized, the residue taken up in 250 μl of 0.05 M sodium acetate, pH 6.2 (RIA-buffer). The cGMP was then estimated using the cGMP RIA-kit from DuPont (NEX-133, NEN Research Products).

Analytical Procedures

The mass spectra were measured with a MAT 90 spectrometer (Finnigan-MAT) in thioglycerol. IR spectra were run in KBr on a Nicolet-FT-IR spectrometer 7199 (Nicolet Instrument Corp.). The UV spectra were recorded on a UVIKON 810 spectrometer (Kontron). The optical rotations were measured on a Perkin-Elmer 241 polarimeter.

The amino acid analyses and the differentiation of asparagine and aspartic acid were performed according to the methods of SPACKMAN *et al.*[13]. The hydrolyses were done with 6 N HCl at 110°C for 24 hours or with 4 N methanesulfonic acid. The determinations of the amino acids in the hydrolysates were carried out on a Liquimat III amino acid analyzer (Kontron).

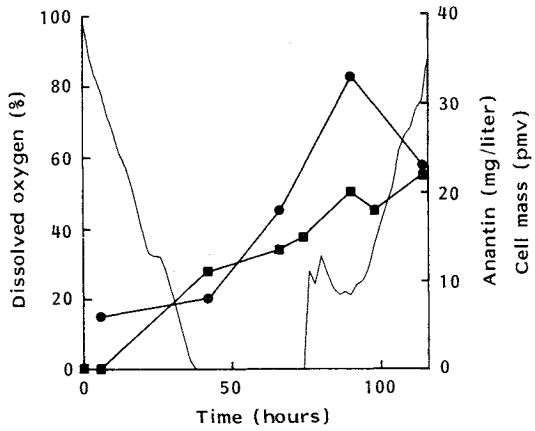
Results and Discussion

Fermentation

A typical time course of a fermentation is shown in Fig. 1. Anantin was found to be stable in the culture broth and most of it was contained in the cell free culture supernatant.

Fig. 1. Time course of a 200-liter fermentation.

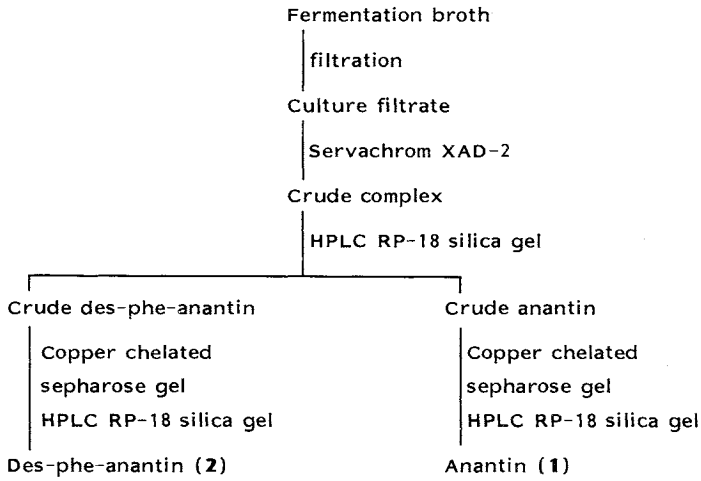
■ Anantin (mg/liter), ● cell mass (pmv), — dissolved oxygen (%).



Isolation

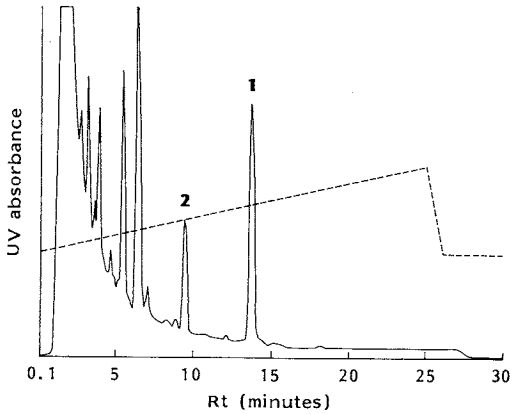
The isolation procedure is shown in Fig. 2. The broth of a 200-liter fermentation was filtered. The culture filtrate (160-liter) was passed through a column of Servachrom XAD-2 (Serva, 15 × 50 cm, particle size: 0.3 ~ 0.9 mm, flow rate: 20 liters/hour). After washing with water (20-liter) and 10% 2-propanol (20-liter), the column was eluted with

Fig. 2. Isolation procedure of anantin (**1**) and des-phe-anantin (**2**).



50% 2-propanol (25-liter). The whole eluate was concentrated and impurities were removed by filtration. The HPLC of this crude product is shown in Fig. 3. Five 200-liter fermentations yielded 12.8 liters of concentrated crude product. Separation of anantin and des-phe-anantin was achieved on reversed phase silica gel. A 3-liter sample of the crude product (52 g dried solids, 2.04 g anantin, 0.77 g des-phe-anantin) was diluted with 3-liter of 50 mM NH_4HCO_3 and was applied on a Prep Pak C-18 silica gel column (Waters, 4.7 × 30 cm, particle size: 55 ~ 105 μm, flow rate: 80 ml/minute). The column was washed with 50 mM NH_4HCO_3 (1.6-liter), 20% MeOH in 50 mM NH_4HCO_3 (1.6-liter) and 40% MeOH in 50 mM NH_4HCO_3 (1.6-liter). With 50% MeOH in 50 mM NH_4HCO_3 (4.0-liter) des-phe-anantin (0.61 g), followed by anantin (1.13 g) were eluted. The active fractions were concentrated *in vacuo*. Further purification of anantin was attained on a column with a copper chelate gel of the type Sepharose(6B)$-O-CH_2-CH(OH)-CH_2-N-(CH_2-COOH)_2$, synthesized from Sepharose CL 6B FF as described in HOCHULI[14] (Pharmacia, 7 × 21 cm, flow rate: 2.4 liters/hour). The column was pre-equilibrated with 50 mM $CuSO_4$ (2-liter), water (2-liter), 50 mM sodium acetate (pH 3.5), being 1 M in NaCl (2-liter), water (2-liter) and 50 mM Na_2HPO_4 (pH 7.5), being 0.5 M in NaCl (2-liter). A sample of crude anantin (1.55 g) in 50 mM Na_2HPO_4 (pH 7.5), being 0.5 M in NaCl, 10% in MeOH (2.2-liter), was applied on this column (flow rate: 1.2 liters/hour). The column was washed with 50 mM Na_2HPO_4 (pH 7.5), being 0.5 M in NaCl (0.8-liter), then with 2 mM imidazole in the same buffer (1.9-liter), and finally anantin was eluted with a gradient of 2 ~ 6 mM imidazole in the same buffer (2.4-liter). The active fractions (1.4-liter) were desalted on a Prep Pak C-18 silica gel column (Waters, 4.7 × 30 cm, particle size: 50 ~ 105 μm, flow rate: 80 ml/minute). The column was washed with water (1.6-liter), with 50 mM NH_4HCO_3 (1.6-liter) and with 40% MeOH in 50 mM NH_4HCO_3 (2.8-liter). Anantin was eluted with a linear gradient of 40 ~ 60% MeOH in 50 mM NH_4HCO_3 (5.2-liter). The active fractions (1.2-liter) were concentrated *in vacuo* and finally lyophilized to give anantin (**1**) as colorless powder (0.65 g). Purification of des-phe-anantin was carried out in a manner similar to that of anantin. Crude des-phe-anantin (0.88 g) was applied on the copper chelate gel column which was washed with 1 mM imidazole in 50 mM Na_2HPO_4 (pH 7.5), being 0.5 M in NaCl (6.2-liter). Des-phe-anantin eluted within the last fractions (1.8-liter). Desalting and final purification on the C-18 silica gel column yielded des-phe-anantin (**2**) as colorless powder (0.57 g).

Fig. 3. HPLC profile of anantin (**1**) and des-phe-anantin (**2**) in a crude fermentation product.



Column: LiChrospher 100 RP-18 (Merck, 125 × 4 mm i.d., 5 μm), mobile phase: 30 ~ 60% acetonitrile containing 5 mM TFA, flow rate; 1.0 ml/minute, detection: UV 228 nm, apparatus: Kontron HPLC system 400.

Dimethyl Ester of Anantin (3)

Anantin (40 mg) was dissolved in MeOH (40 ml) containing a few drops of 96% H_2SO_4. The mixture was held for 65 hours at room temperature. The reaction mixture was diluted with 120 ml of water and applied on a Nucleosil C-18 column (Macherey-Nagel, 2 × 25 cm, 10 μm particle). The column was washed with 30% acetonitrile, 5 mM in TFA. The product was eluted with 50% acetonitrile, 5 mM in TFA.

Table 1. Physico-chemical properties of anantin (**1**) and des-phe-anantin (**2**).

	Anantin (**1**)	Des-phe-anantin (**2**)
Molecular formula	$C_{90}H_{111}N_{21}O_{24}$	$C_{81}H_{102}N_{20}O_{23}$
FAB-MS (*m/z*)	1,870.9 (M+H)	1,723.6 (M+H)
	Calcd 1,870.8	Calcd 1,723.7
$[\alpha]_D^{20}$	+12°	+26.6°
	(*c* 0.9, MeOH)	(*c* 1.01, MeOH)
UV λ_{max}^{MeOH} nm (ε)	279 (6,989)	279 (6,841)
TLC Rf value[a]	0.63	0.52
Color reactions:		
Reindel-Hoppe	Positive	Positive
Ninhydrin	Negative	Negative
Ehrlich	Positive	Positive

[a] Silica gel 60 F_{254} (Merck), BuOH - AcOH - water (80 : 20 : 20).

Table 2. Amino acid composition of anantin (**1**) and des-phe-anantin (**2**).

Amino acid	Anantin (**1**)		Des-phe-anantin (**2**)	
	Amount found[a]	Amount calcd	Amount found[a]	Amount calcd
His	1.03	1	1.01	1
NH_3	1.40		1.06	
Asx[b]	3.00	3	3.00	3
Ser	0.90	1	0.87	1
Gly	5.00	5	5.00	5
Ile	2.01	2	2.01	2
Tyr	1.01	1	1.01	1
Phe	2.93	3	2.00	2
Trp	0.98	1	1.01	1

[a] Amino acid analyzer.

[b] The value for Asx was set as 3. Further analysis showed the composition of Asx to be 2 Asp plus 1 Asn.

Fig. 4. IR spectra of anantin (**1**) and des-phe-anantin (**2**) (KBr).

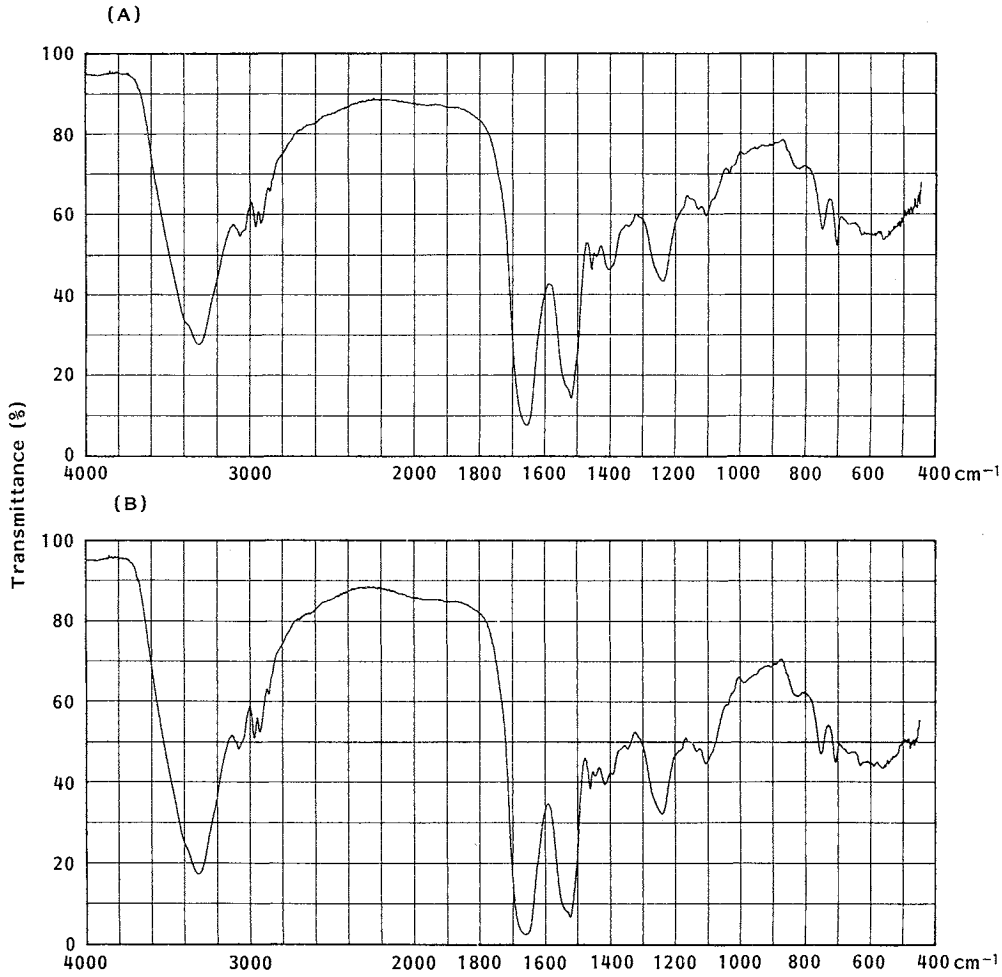
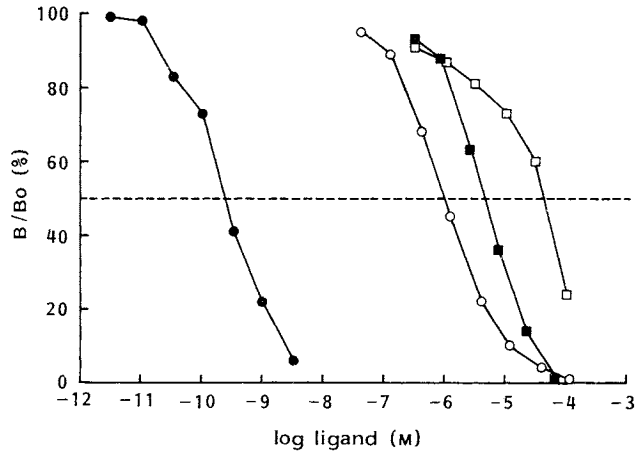


Fig. 5. Inhibition of rat ^{125}I-ANF binding to bovine adrenal cortex receptors.

□ Des-phe-anantin, ■ dimethyl ester, ○ anantin, ● APIII.



Membranes (containing 60 μg protein) were incubated with 20 pM (25,000 cpm) of radioligand with increasing amounts of the indicated ligands, APIII, atriopeptin III or rat ANF (103–126).

The peak fractions were concentrated *in vacuo* and lyophilized and the dimethyl ester of anatin (**3**) was obtained as white powder (31 mg).

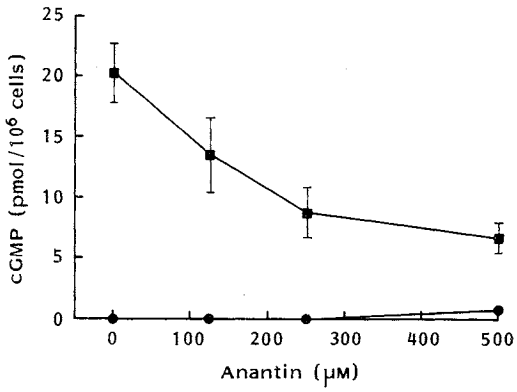
FAB-MS of **3**: *m/z* 1,898.9 $(M+H)^+$, calcd for $C_{92}H_{116}N_{21}O_{24}$ 1,898.8; IR ν_{max} (KBr) cm^{-1} 3299, 2994, 2881, 1733 (sh), 1669, 1520, 1438, 1402, 1205, 1134, 836, 801, 745, 701.

Physico-chemical Properties

The physico-chemical data of anantin (**1**) and des-phe-anantin (**2**) are shown in Table 1. Dominant absorptions at 1655 and 1515 cm^{-1} in the IR spectra (Fig. 4) indicated these compounds to be peptides. The amino acid analysis (Table 2) showed anantin to be composed of 17 amino acids. Based on FAB-MS the elemental composition $C_{90}H_{111}N_{21}O_{24}$ of anantin is proposed. Anantin was esterified to the dimethyl ester of anantin (**3**) with acidic methanol, suggesting the presence of two carboxylic acid residues. Compound **3** could be hydrolyzed with dilute ammonia to give anantin again.

Fig. 6. Effects of anantin on cGMP in bovine aorta smooth muscle cells.

■ +APIII (10 μM), ● −APIII.



The cells were incubated with various concentrations of anantin in presence or absence of 10^{-5} M APIII (atriopeptin III, rat ANF (103-126)).

Des-phe-anantin (**2**) is closely related to anantin. It is composed of 16 amino acids, the missing amino acid being phenylalanine.

The structures of anantin and des-phe-anantin as determined by peptide sequencing and 2D NMR investigations are described in Wyss *et al.*, succeeding paper[15].

Antibiotic Activity

Anantin has been tested for antimicrobial activity against a broad variety of bacteria and fungi. No antibiotic acitivity has been found (data not shown).

Effect of Anantin in the ANF-Receptor Assay

Anantin inhibited the binding of ^{125}I-ANF by 50% (IC_{50}) at 1.0 μM, which is 4,000-fold less potent than rat ANF (103-126) (atriopeptin III, Fig. 5). Computer calculations of the competition curves based on a true Mass Action Law multiligand/multisite algorithm[16] indicated that the curves are best explained by a one-site/one-ligand model for both ANF and anantin. The K_d values were 18.9 pM and 610 nM and the receptor densities 4.8 pmol/mg protein and 4.3 pmol/mg protein, respectively. The fact that both anantin and ANF react only with one receptor site with the same density suggests a competitive interaction of anantin with ANF at the ANF-binding site. Interestingly, des-phe-anantin (**2**) was 50-times less potent in the binding assay than anantin (IC_{50} 58 μM), whereas the dimethyl ester (**3**) was only 2.5-times less potent (IC_{50} 2.5 μM).

Effect of Anantin on Intracellular cGMP

When anantin was incubated with bovine aorta smooth muscle cells, no change in intracellular cGMP was observed. However, when ANF was added 5 minutes after preincubation with anantin at various concentrations, the increase in intracellular cGMP (from <0.25 to 20 pmol/million cells) was found to be dose-dependently and significantly inhibited by anantin (Fig. 6). Hence, anantin behaved as a functional ANF-antagonist which was devoid of agonistic activity.

Acknowledgments

We acknowledge the excellent technical assistance of JOSIANE REIN and PAUL MORY (biochemical assays), LAURENCE HILFIKER (cell cultures), J. JOERGER (downstream processing) and B. KÜHNIS (fermentation). We are grateful to Mr. M. MANNEBERG for the amino acid analyses, to Mr. A. BUBENDORF for IR spectra and to Dr. W. VETTER for the mass spectra. Furthermore, we appreciate the collaborative work in the preparation of cells by Dr. MARTINE CLOZEL.

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