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Receptor binding, cGMP stimulation and receptor desensitization by atrial natriuretic peptides in cultured A10 vascular smooth muscle cells

Dieter Neuser and Peter Bellemann

ATR-Hypertension, Research Center, Bayer AG, PO Box 10 17 09, D-5600 Wuppertal 1, FRG

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Receptor characteristics for atrial natriuretic peptides (ANP) were demonstrated in the permanent tissue culture system of vascular smooth muscle cell (VSMC) line. ^{125}I-ANP exhibited reversible and saturable binding to A10 rat VSMC, and the equilibrium dissociation constant, K_d was 157 pM and maximal binding capacity, B_{max} amounted to 115 fmol/mg of protein. Binding of the ^{125}I-ligand was highly specific for certain potently displacing ANP analogues with inhibition constants (K_i values) in the nano- or even subnanomolar concentration range. Pretreatment of VSMC with ANP yielded receptor desensitization to one half of the ANP receptor density, and supports the conclusion of receptor autoregulation by ANP in A10 VSMC. The receptor coupled intracellular cGMP system was stimulated, and thus demonstrates the application of A10 cells as a model for the study of ANP receptor interaction involved in vascular smooth muscle relaxation.

Atrial natriuretic peptide *Receptor binding* *cyclic GMP* *cyclic AMP* *Receptor desensitization* *(Vascular smooth muscle, A10 cell, CRL 1476 cell)*

1. INTRODUCTION

Mammalian atria contain powerful natriuretic and vasoactive peptides (review [1]) termed collectively as atrial natriuretic peptides (ANP). They represent a new class of hormones synthesized in and released from mammalian atria into the circulation dependent on the volume status [1–2], and aside from their key role in regulating fluid volume and water-electrolyte balance ANPs also exert a potent effect on blood pressure [1]. Recently, ANP was shown to bind to primary cultures of smooth muscle cells derived from rat or bovine aorta explant [4–6]. The present report documents binding characteristics of ^{125}I-ANP, cGMP stimulation and ANP receptor down-regulation using the A10 vascular smooth muscle permanent cell (VSMC) line [7].

Correspondence address: P. Bellemann, ATR-Hypertension, Research Center, Bayer AG, PO Box 101709, D-5600 Wuppertal 1, FRG

2. MATERIALS AND METHODS

2.1. *Preparation of radiolabelled rat ANP*

Iodination of rat ANP III was carried out as described [2] using the chloramine T method. ^{125}I-ANP was purified by reverse-phase chromatography on RP 18 with an acetonitrile/TFA (0.1%) gradient [2]. The resulting specific activity of the radioligand was about 1900 Ci/mmol.

2.2. *Cell cultures*

Cells were cultured in Dulbecco's MEM containing FCS (10%), L-glutamine (6.8 mM), Hepes (20 mM) and penicillin/streptomycin (50 IU/ml, each) using an atmosphere of CO_2/air (1:19). At confluence cells were trypsinized and subcultured in tissue culture flasks. For receptor binding and

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cyclic nucleotide stimulation experiments cells were removed by trypsinization (0.05%), counted and seeded onto 24-well tissue culture clusters of costar (Greiner, Nürtingen, FRG) at a density of 2 $\times$ 10^5 cells/well. Experiments were started after cells became adherent for about 16 h.

2.3. *Down-regulation of vascular ANP receptors*

After the inoculation period A10 VSMC were incubated in fresh Dulbecco's MEM culture medium without or with unlabelled ANP (0.01 μM or 1 μM) at 37°C for 20 h. ANP receptor affinity and capacity were then estimated by radioligand binding assays as given below.

2.4. *Binding assay*

Cultured A10 cells were washed twice with warmed binding medium (Dulbecco's MEM containing 0.05% BSA, pH 7.4 at 37°C). In a final volume of 1 ml, cells were incubated at 37°C in binding medium containing the indicated concentration of ^{125}I-ANP and various additives, e.g., peptides as displacer. After the indicated time intervals, the reaction was terminated by rapidly aspirating the incubation fluid and washing the monolayers twice with 2 ml of ice-cold binding medium. Cells were then disintegrated by 0.5 ml of NaOH/Triton X-100 (0.2 M/0.1%), and cellular radioactivity was measured by gamma-scintillation spectrophotometry. Protein was measured as described [8] with serum albumin as the standard. Data were calculated and plotted according to Scatchard, and displacement experiments were analyzed with computer programmes.

2.5. *Determination of cellular cGMP and cAMP*

A10 cultured VSMC were washed twice with assay medium (Dulbecco's MEM containing 0.05% BSA) and then incubated in a final volume of 0.5 ml of assay medium with or without isobutylmethylxanthine (0.1 mM) for 5 min. ANP or their fragments were added and further incubated for the indicated time intervals. Incubation was terminated by removing the assay medium and by adding 2 ml of trichloroacetic acid (10%). The disintegrated cell suspensions were aspirated and centrifuged. The supernatant was extracted with four portions of ethylether, and the aqueous phase was finally lyophilized overnight. The residue was dissolved in Na-acetate buffer (50 mM, pH 6.2) and cGMP and cAMP were then determined according to the technical guide of the assay kits (NEN).

2.6. *Materials*

The rat aortic smooth muscle cell line A10 (CRL 1476) was obtained from the American Type Culture Collection, ATCC (Bethesda, MD). Tissue culture media and admixtures were purchased from Boehringer Mannheim or Gibco, Karlsruhe, FRG. Na^{125}I, cGMP and cAMP kits were from New England Nuclear (Boston, MA), ANP and their fragments were from Peninsula Labs; all chemicals used were reagent grade.

3. RESULTS AND DISCUSSION

^{125}I-ANP interacted with membrane fractions from bovine adrenal cortices; however, specific binding in this preparation was low and ranged between 15 and 20%, only. Thus, intact A10 VSMC in monolayer cultures were used for receptor binding studies. Specific binding to cultured VSMC was linearly proportional to cell density up to 5 $\times$ 10^6 cells/well; thus, receptor binding studies were carried out in the linear range using 2 $\times$ 10^5

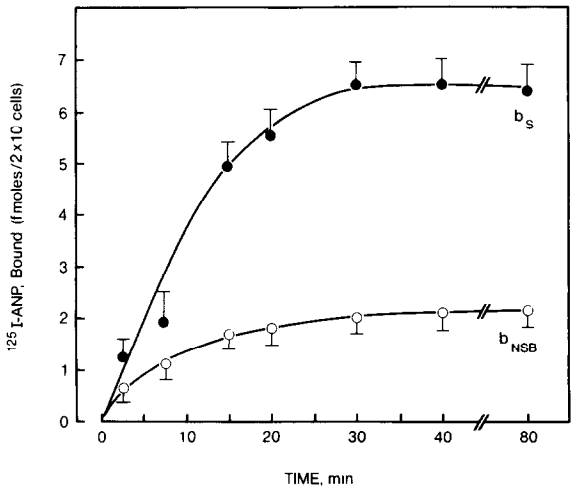

Fig.1. Time dependence of ^{125}I-ANP binding to cultured A10 vascular smooth muscle cells. The figure shows the time course of ^{125}I-ANP association to intact cell cultures as specific (●) and non-specific (○, presence of 1 μM ANP) binding. Results presented are the mean ± SD of 6 separate experiments.

cells/well (approx. 200 μg of protein). Addition of 1 μM ANP (non-specific binding) to the assay displaced 75–90% of the total ^{125}I-ANP binding, and the amount of radioactivity adherent to the plastic surface ranged between 1.7 and 2.6%, only. As checked by HPLC [2] the iodinated radioligand remains stable under the experimental conditions reported here.

Specific binding (b_s) of ^{125}I-ANP to A10 VSMC was a time-dependent process (fig.1), and steady-state values were reached after 30 min and remained constant over a period of more than 2 h. Binding of ^{125}I-ANP in the presence of 1 μM unlabelled ANP (non-specific binding, b_{NSB}) also occurred time-dependently and amounted to 20–30% of specific binding.

The specific binding of ^{125}I-ANP was saturable and plateaus were achieved between 250 and 300 pM of radioligand (fig.2). Scatchard analyses of the saturation isotherms (fig.2, inset) revealed a straight line indicating the presence of a single binding site. The equilibrium dissociation constant, K_d was 157 ± 29 pM (n = 6; $r > 0.95$), and the number of receptor binding sites (capacity), B_{max} equivalent to 115 ± 8 fmol/mg protein (n = 6; $r > 0.95$). Similar results were reported for ANP binding to crude rabbit aortic membranes [9], and to primary cultures of smooth muscle cells from bovine and rat aorta [4,6].

Pretreatment of A10 VSMC with unlabelled ANP for 16 h resulted in a marked reduction of the total number of receptors without major alteration in the affinity. Scatchard analyses of the ^{125}I-ANP specific binding to A10 cells without ANP pretreatment showed a receptor capacity of about 120 fmol/mg protein. The reduction of receptor density was gradual and related to the ANP concentration applied (fig.3): 10^{-8} M and 10^{-6} M ANP pretreatment diminished ANP receptor density to 100 and 60 fmol/mg protein, respectively. Data presented substantiate the conclusion of receptor autoregulation by ANP in smooth

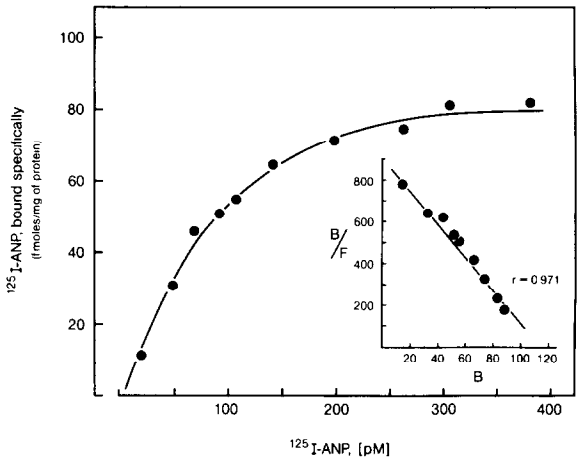

Fig.2. Saturation isotherm of ^{125}I-ANP binding to cultured A10 vascular smooth muscle cells. Cells were incubated at least in triplicate with ^{125}I-ANP, 10–380 pM, in the presence or absence of unlabelled ANP. Specific binding (●, fmol/mg of protein) was calculated as the difference between total binding and that not displaced by excess (1 μM) of ANP. The latter (non-specific binding) ranged between 10 and 25% (see text). Inset: Scatchard analysis of specific binding data (B, bound ligand; F, free radioligand) revealed linearity and indicated one binding site with an equilibrium dissociation constant K_d = 0.13 nM (reciprocal of the slope value) and maximal binding, B_{max} of 120 fmol/mg of protein (r = 0.971; intercept on abscissa). The experiment was repeated several times with different cell preparations and yielded identical results ($r > 0.95$).

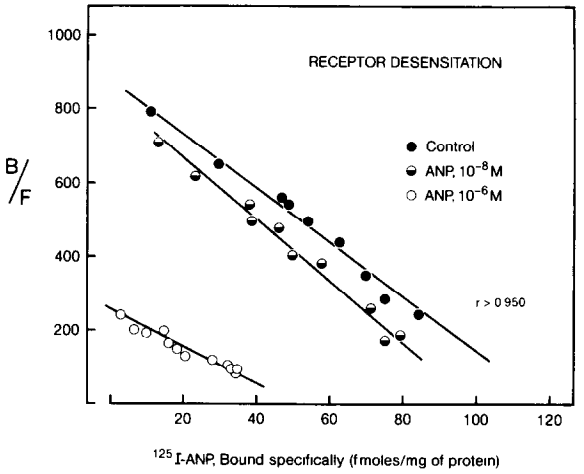

Fig.3. ANP receptor desensitization. Scatchard analyses of ^{125}I-ANP specific binding to cultured A10 vascular smooth muscle cells pretreated for 16 h without (control) or with unlabelled ANP (0.01 μM; 1 μM ANP). Results show no major changes in receptor affinity, but binding capacity was reduced concentration-dependently from 120 fmol/mg of protein (●, control) to 100 (○, 0.01 μM ANP) or 60 fmol/mg of protein (○, 1 μM ANP), respectively. Data are representative results of 3 separate experiments.

muscle cells [10,11], and consequently receptor desensitization may also occur under certain pathophysiological conditions linked to exaggerated ANP levels in the blood circulation.

The pharmacological manner of the ^{125}I-ANP receptor sites is demonstrated by displacement studies with various ANP. Fig.4 presents an example of such competitive ^{125}I-ANP binding experiments in A10 VSMC using unlabelled ANP derivatives. Rat ANP and α-human ANP (1–28) both inhibited the binding of ^{125}I-ANP to the receptor concentration-dependently with apparent inhibition constants (K_i values) of 71.5 ± 8 pM (n = 8) for both peptides.

Different atrial natriuretic peptides and fragments generally inhibit the binding of ^{125}I-ANP to A10 VSMC (table 1). Shortening of the C-terminal amino acid sequence, e.g., ANP I, ANP II resulted in reduced binding of the peptides to the ANP receptor. In contrast, fragment (7–28), a peptide without the N-terminal sequence, still exerted a similar inhibitory activity to ANP III, r-ANP (1–28) and h-ANP (1–28). Fragments without the intramolecular disulfide bond, e.g., fragments (13–28), (18–28), (1–11), totally lost the ability to interact with the ANP receptor. These structure-activity relationships correspond well with the vascular reactivity of various ANP derivatives in the rabbit aortic strip preparation [12].

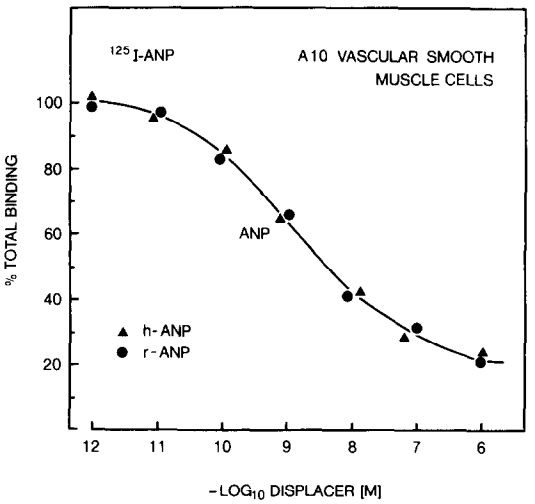

Fig.4. Displacement experiments using ^{125}I-ANP (< 100 pM) and the unlabelled peptides, rat ANP (●) and human ANP (▲) dissolved in tissue culture medium. Total binding to cultured A10 vascular smooth muscle cells is plotted against $-\log_{10}$ of displacer concentration [M]. Data are the means of 3–5 separate experiments in triplicate with less than 5% standard deviation.

Table 1

Inhibition of ^{125}I-ANP binding and effect on cyclic nucleotides by various atrial natriuretic peptides in cultured A10 vascular smooth muscle cells

Peptides	Inhibition (%)	cGMP (fmol/2 × 10^5 cells)	cAMP (pmol/2 × 10^5 cells)
ANP I	40	n.d.	n.d.
ANP II	60	n.d.	n.d.
ANP III	85	300	18
ANP (1–28)	90	290	19
h-ANP (1–28)	90	320	16
Fragment 7–28	85	320	–
Fragment 13–28	0	55	–
Fragment 18–28	0	65	–
Fragment 1–11	0	58	–
Control	–	60	21

Inhibition of ^{125}I-ANP binding and cyclic nucleotide assays were performed as described in section 2. Results presented are the mean of 6 separate experiments with less than 10% SD

The functional coupling of the ANP receptor to intracellular target sites is demonstrated by measuring cellular levels of cGMP and cAMP in the presence of several ANP analogues. Rat ANP, α-human ANP (1–28) and fragment (7–28) enhanced cGMP concentration in A10 VSMC to almost equal amounts, whereas intracellular cAMP levels remain unaffected by the compounds. Stimulation of cGMP by atrial natriuretic peptides was recently reported also in rat and bovine aortic as well as in endothelial cells [4,6], and thus suggests the particular involvement of the intracellular cGMP level in its vasorelaxant effect, but not the cAMP system.

In summary, the results of the binding characteristics and the subsequent stimulation of the intracellular cGMP system presented here support the conclusion that the modulation of specific receptive sites for ANP by various ANP derivatives and the receptor coupled intracellular events are studied well in the permanent tissue culture system of A10 vascular smooth muscle cells.

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