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# DOWN-REGULATION OF ATRIAL NATRIURETIC FACTOR RECEPTORS AND CORRELATION WITH cGMP STIMULATION IN RAT CULTURED VASCULAR SMOOTH MUSCLE CELLS

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The relationship between the binding of $^{125}$I-labeled rat ANF and the responsiveness in cGMP production of ANF receptors were examined in cultured rat thoracic smooth muscle cells after preexposure with the peptide. Binding assays of $^{125}$I-labeled ANF showed a specific, reversible and saturable binding with a $K_D$ value of 3.1 $\pm$ 0.3 $10^{-10}$ M and a maximum binding ($B_{max}$) of 240 $\pm$ 30 fmol/$10^6$ cells. Pretreatement of the cells with increasing concentrations of unlabeled ANF ($10^{-9}$ M to $10^{-7}$ M) resulted in a dose-dependent decrease of the number of binding sites without a change in the affinity. This effect was clearly associated with a desentization of ANF-induced cGMP production. © 1987 Academic Press, Inc.

Atrial natriuretic factor (ANF) represents a new class of peptidic hormones which possess potent natriuretic diuretic and vasoactive properties (reviewed in ref. 1,2). Specific ANF receptors which presumably mediate these effects have been localized in various vascular tissues including aorta of various species (3,4), mesenteric and renal arteries (5), pulmonary artery and vein (4), and brain microvasculature (6). ANF receptor occupancy has been correlated with an elevation of intracellular cyclic GMP (cGMP)(7). Recently, ANF was shown to bind in cultured vascular smooth muscle cells to specific receptors which are functionally coupled to a guanylate cyclase system (8,9,10,11). Moreover these receptors can be "down-regulated" following prior exposure to ANF (10,12). The present document reports the effects of exposure of rat aortic smooth muscle cells to increasing concentrations of rat ANF (rANF) on receptor regulation and its consequence on ANF-induced cGMP production.

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## MATERIALS AND METHODS

### Cell culture

Vascular smooth muscle cells were prepared according to the method of Chamley et al. (13) by enzymatic (elastase and collagenase) digestion of 10 weeks old male Wistar rats thoracic aorta. Vascular smooth muscle cells were cultured in Dubelcco's modified Eagle medium (DMEM) containing 10 % fetal calf serum (FCS), 1 % glutamine and 1 % antibiotics (penicillin, streptomycin, Fungizone) at 37°C in a 95% air-5% $CO_2$ humidified atmosphere. Vascular smooth muscle cells were subcultured by 0.05-0.02 % Trypsin-EDTA and used for experiments between 2th and 5th passage.

### Down regulation of vascular ANF receptors

Confluent cells ($10^6$ cells/dish) were preincubated in 2 ml DMEM containing 0.05 % bovine serum albumin (BSA), 1 % glutamin and 1 % antibiotics with or without rat ANF at different concentrations for 24 hours in the incubator at 37°C.

### Binding assay of ANF

After preincubation in 6-well dishes, the cells were washed three times with 2 ml of DMEM containing 0.05 % BSA (binding buffer) at 37°C. No ANF-like immunoreactivity was detected in the medium after the three washes. Saturation binding experiments were performed with concentrations of $^{125}$I-ANF ranged from 0.05 to 4 nM in binding buffer. Non-specific binding was determined in the presence of 0.5 µM of unlabeled ANF. After 30 min incubation at 37°C, the cells were washed four times with 2 ml of ice-cold binding buffer and then solubilized in 1 ml of 1 N NaOH. Aliquots of 900 µl were assayed for radioactivity in a LKB γ counter with 70% efficiency. $K_d$ and $B_{max}$ values were calculated according to the Scatchard method and analysed with a computer programme (14).

### Determination of intracellular cGMP assay

After preincubation, the cells were washed 3 times with 2 ml of DMEM at 37°C. Then, they were preincubated for 5 min with $2.10^{-4}$ M isobutylmethylxanthine (IBMX) at 37°C and stimulated with or without ANF at various concentrations for 5 min. Rapid aspiration and addition of ice-cold 0.1 N HCl stopped the reaction (15). cGMP was determined by a radioimmunoassay after acetylation (Kit New England Nuclear : NEX - 133).

### Drugs

The radiolabeled ANF ($^{125}$I-ANF) was the 3-[$^{125}$I]iodotyrosyl derivative of rat ANF [Amersham about 2000 Ci/mmol (about 74 TBq/mmol)]. Synthetic rat ANF (rANF) corresponding to the sequence Ser-99 to Tyr-126 [rANF (1-28)] was purchased from Novabiochem (EMA, France) as Atriopeptin III [rANF (5-28)] and Atriopeptin I [rANF (5-25)]. Adrenalin, Angiotensin II, Met and Leu-enkephalin, insulin, Arg-Vasopressin and IBMX were purchased from SIGMA and PAF-acether from Calbiochem (Switzerland).

## RESULTS

### Binding of $^{125}$I-ANF to rat vascular smooth muscle cells

$^{125}$I-ANF bound to a single class of high affinity binding sites in rat aortic smooth muscle cells (Fig. 1). The binding fulfills many of the criteria for specific ANF receptor recognition sites. It is specific, rapid, reversible and saturable. The non-specific binding was between 10 % and 20 % of total binding. Steady state values were reached after 20 min and remained stable over a 90 min period. The specificity of ANF binding was assessed by

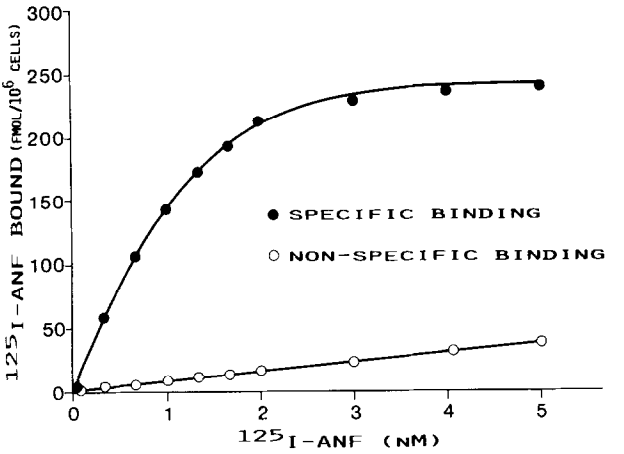


Fig. 1 . Typical saturation curve of $^{125}$I-ANF binding to rat vascular smooth muscle cells. Cultured rat aortic smooth muscle cells were incubated at 37°C for 30 min with increasing concentration of $^{125}$I-ANF. Specific (●) and non-specific (○) binding was determined as described under Material and Methods.

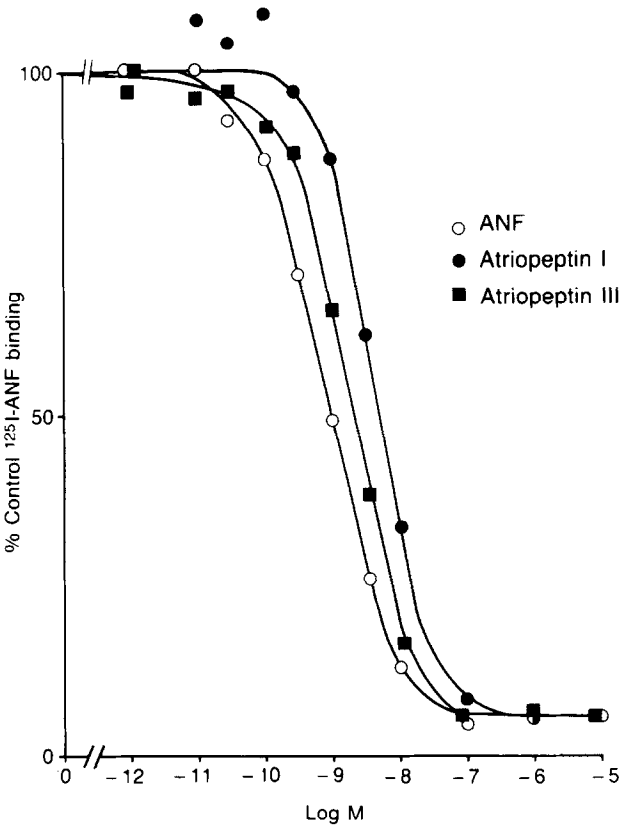


Fig. 2 . Inhibition of $^{125}$I-ANF binding to rat vascular smooth muscle cells. Cultured cells were incubated at 37°C for 30 min with 3.6 $10^{-10}$ M $^{125}$I-ANF in the presence of increasing concentrations of unlabeled rANF (1-28) (○), Atriopeptin I (●) and Atriopeptin III(■). Each point is the mean of triplicates.

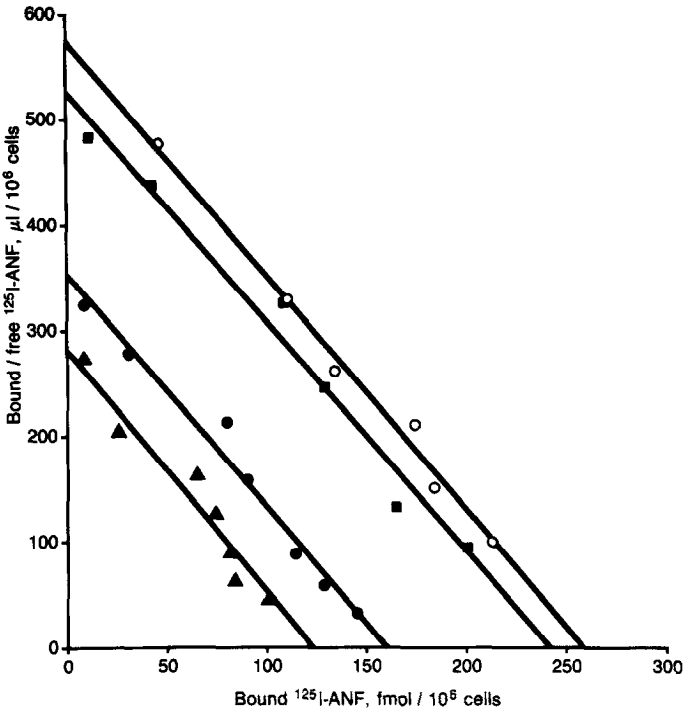


Fig. 3 . Scatchard analysis of a typical binding experiment of $^{125}$I-ANF to rat aortic smooth muscle cells preincubated with $10^{-9}$ M (■), $10^{-8}$ M (●) and $10^{-7}$ M (▲) or without (○) unlabeled rANF. For this experiment the $K_d$ value is 4.3 $10^{-10}$ M and the $B_{max}$ values are 260, 240, 160 and 120 fmol/$10^6$ cells for control, rANF $10^{-9}$ M, rANF $10^{-8}$ M and rANF $10^{-7}$ M, respectively.

displacing $^{125}$I-ANF with unlabeled rANF (1-28) and related peptides. Displacement experiments using 3.6 $10^{-10}$ M $^{125}$I-ANF showed that rANF (1-28) inhibited the binding with an $IC_{50}$ of 7.9 $10^{-10}$ M, Atriopeptin III with an $IC_{50}$ of 1.6 $10^{-9}$ M and Atriopeptin I, the less active with an $IC_{50}$ of 4.5 $10^{-9}$ M (Fig. 2). Furthermore an absence of competition was observed with 1 to 10 µM of unrelated compounds such as, Ang II, Insulin, Arg-Vasopressin, Met-Enkephalin, Leu-Enkephalin, PAF-acether and adrenalin. The $K_d$ and $B_{max}$ calculated from five saturation experiments by Scatchard analysis were 3.1 ± 0.3.$10^{-10}$ M and 240 ± 30 f/mol $10^6$ cells, respectively.

Similar results were reported for ANF binding from cultured vascular smooth muscle cells of bovine and rat aorta (8,10) and from the permanent A10 vascular smooth muscle cells line (16).

Down regulation of ANF receptor in rat vascular smooth muscle cells

Pretreatment of the cells with rANF for 24 hours resulted in a decrease of the number of binding sites without a significant change in the affinity. Moreover a concentration dependent effect was established in our experiments: pretreatment of the cells with increasing concentrations of unlabeled rANF

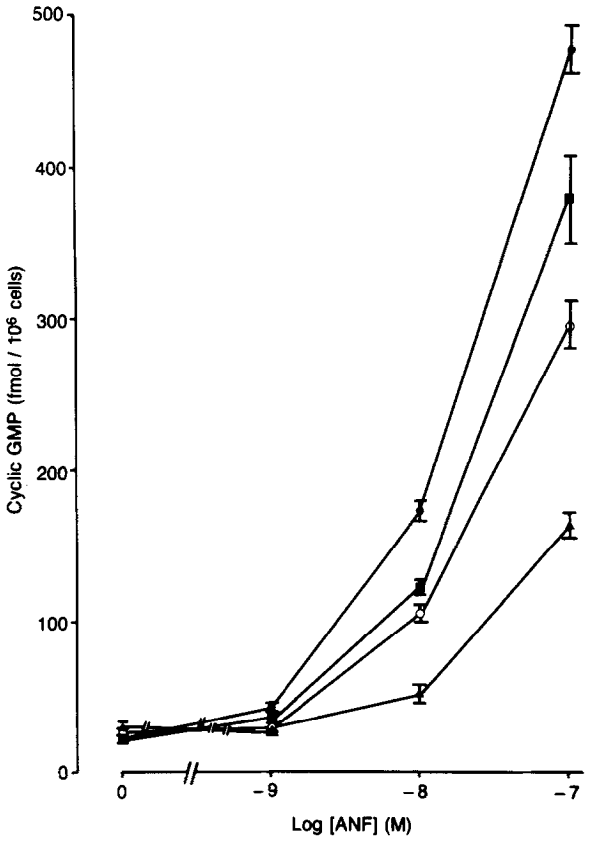


Fig. 4 . Effects of rANF on cGMP production after preexposure of vascular smooth muscle cells with different concentrations of rANF. Confluent cells ($10^6$ cells) were preincubated for 24 hrs at 37°C in the absence (●) or presence of $10^{-9}$ M (■); $10^{-8}$ M (○); $10^{-7}$ M (▲) unlabeled rANF. Concentration of intracellular cGMP were determined by radioimmunoassay after acetylation in presence of IBMX ($2.10^{-4}$ M) following an incubation of 5 min. The curves represent the mean of four different experiments in which each point was made in quadriplicates.

$10^{-9}$ M, $10^{-8}$ M and $10^{-7}$ M diminished the density of receptors to 7 %, 47 %, 55 %, respectively (mean of 3 experiments) (Fig. 3).

Concerning the time course of the down-regulation of the receptors, it was observed that the maximal effect occurred in 20 minutes suggesting an internalization of the receptors and persisted at 24 hours if the medium was not changed. Following removal of external rANF by an extensive washing and a reincubation of the cells in DMEM, a recovery of 90 % of the initial binding capacity appeared after 6 hours (data not shown) indicating presumably the requirement of protein synthesis.

Stimulation of cGMP after down-regulation

As a functional coupling of ANF receptors to particulate guanylate cyclase exists in cultured vascular smooth muscle cells (11), and as cGMP has

been proposed as a second messenger, it was therefore interesting to examine the consequence of the preexposure of the cells by the peptide on cGMP stimulation. In control cells, as well as in pretreated cells, rANF ($10^{-9}$, $10^{-8}$ or $10^{-7}$ M) increased cGMP synthesis in a dose-dependent manner (Fig. 4).

However, the stimulation of production of cGMP of the cells whose ANF receptors were down regulated was markedly reduced according to the rANF concentration pretreatment (Fig. 4). For example, cGMP production induced by rANF ($10^{-7}$ M) was decreased by 20 %, 38 % and 65 % in preincubated cells with rANF $10^{-9}$, $10^{-8}$ and $10^{-7}$ M, respectively.

## DISCUSSION

The present study shows that preexposure of rat vascular smooth muscle cells to rANF leads in a dose-dependent manner to a diminution of ANF binding sites without modifying the affinity. These results are consistent with previous reports on ANF receptor down-regulation (9,10). In our experiments, the characterization and the regulation of ANF receptors have been realized using rANF [rANF (1-28)]; Atriopeptin III and Atriopeptin I corresponding to the sequence of rANF (5-28) and rANF (5-25) were also effective to displace $^{125}$I-ANF binding. In accordance with the results obtained by other groups (12,17,18), these analogs presented only minor differences in apparent binding affinity with the parent compound, Atriopeptin I being the less potent. Moreover, our study demonstrates a close correlation between the diminution of the number of binding sites after down-regulation and the subsequent response in cGMP production to a further stimulation with rANF. These results suppose that down-regulation of ANF receptors induces a desensitization of ANF-mediated cGMP production. Recently, although Scatchard analysis of binding data suggest the existence of only one class of ANF binding sites, the presence of multiple subtypes of ANF receptors have been proposed in vascular smooth muscle cells (17) as well as in endothelial cells (18).

Because of a lack of correlation between receptor binding affinity for ANF analogs and their potency to stimulate cGMP, it was supposed that one type of ANF recognition sites is coupled to guanylate cyclase and one other, the more abundant is uncoupled to this second messenger system (17, 18).

Our results show that down-regulation of ANF receptors by rANF does not allow to differentiate these two populations of receptors. This is not surprising since rANF which binds to ANF receptors and increases cGMP well interact with the two receptors subtypes (18) and could therefore down-regulate both of them. However, different results were recently obtained by Hirata et al. (19) who found in rat aorta smooth muscle cells that human-ANF which has very similar binding characteristics as rANF stimulated cGMP

formation in pretreated cells for 24 hrs with 1.6 $10^{-7}$ M of human ANF to the same extent as in control cells. This suggested to their authors that only ANF receptors which are uncoupled to a guanylate cyclase would be sensitive to down-regulation. Our present data do not support this assumption and demonstrate that down-regulation of ANF receptors in rat vascular smooth muscle cells is associated in a dose-dependent manner with a desensitization of ANF-mediated cGMP production.

While this work was in progress, similar observations on a concomittant diminution and desensitization of ANF receptors recognition sites were reported on AtT-20 corticotropin-secreting tumor cell line after down-regulation (20). These results and our results tend to support that in homologous down-regulation, the observed diminution of ANF binding sites correlates to a decrease in cGMP responsiveness.

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