Volume 228, number 2, 290-294 FEB 05583 February 1988

Functional multiplicity of atrial natriuretic peptide receptors on cultured rat Leydig tumor cells

George P. Vlasuk, Karen E. Arcuri, Terry M. Ciccarone* and Ruth F. Nutt*

Departments of Biological Chemistry and **Medicinal Chemistry, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, USA*

Received 23 December 1987

Native rat atrial natriuretic peptide (NANP) was shown to bind with high affinity and to increase intracellular levels of cGMP in cultured rat Leydig tumor cells. A linear analog of NANP which lacks the disulfide-linked bridge structure also bound with high affinity but did not increase levels of intracellular cGMP or antagonize the increase of this cyclic nucleotide by NANP. These data are consistent with the existence of two functional subpopulations of ANP receptors on cultured rat Leydig tumor cells; one which is capable of activating guanylate cyclase and one which is not linked to this enzyme.

Atrial peptide; Linear atrial natriuretic peptide analog; Guanylate cyclase

1. INTRODUCTION

Atrial natriuretic peptide (ANP), which is synthesized and secreted by atrial myocytes, has been shown to exhibit potent natriuretic, diuretic and vasorelaxant activity (review [1]). The biological activities of ANP are manifested through its interaction with specific high-affinity receptors which have been characterized in a variety of different tissues and cultured cell systems [2].

The mechanism of signal transduction following ANP receptor occupation is unknown. However, ANP has been demonstrated to increase the levels of intracellular cGMP in several different cell types and tissues exclusively through the activation of the particulate form of guanylate cyclase [3]. A functional multiplicity of the ANP receptor in several cultured vascular cell lines has been demonstrated [4–6]. In addition, purification of at least two molecular forms of high-affinity ANP-binding proteins from vascular [7] and adrenal tissue [8] has recently been reported.

The present study documents the existence of high-affinity ANP receptors on a cultured rat Leydig tumor cell line. In addition, we present data on the dissociation of ANP receptor binding and cGMP formation with native and a linear analog of ANP which suggests that the ANP receptor population on these cells may exist in two different functional subpopulations; one which is capable of activating guanylate cyclase and one which is not.

Correspondence address: G.P. Vlasuk, Department of Biological Chemistry, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, USA

Abbreviations: cGMP, guanosine cyclic 3′,5′-monophosphate; NANP, native atrial natriuretic peptide; LANP, linear atrial natriuretic peptide

2. MATERIALS AND METHODS

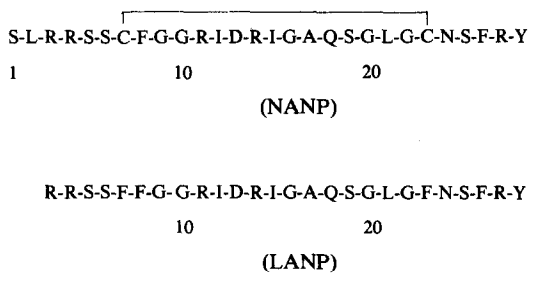
2.1. *Rat Leydig cell culture*

The cells used in these experiments (LC540) were derived from a rat Leydig cell testicular tumor and were obtained from the American Type Culture Collection (CCL43). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM Hepes, pH 7.4. All culture media and supplements were purchased from Gibco. Cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Media were changed twice weekly and cells were passaged from confluent cultures at a 1:5 dilution following removal with trypsin/EDTA.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.2. *ANP-binding studies*

The synthetic ANP peptides which were used in the experiments are the native 28-amino-acid rat ANP (NANP) and a 26-amino-acid linear analog (LANP) which lacks the 17-membered disulfide-linked bridge structure. Their structures are shown below:



NANP was obtained from Bachem while LANP was prepared by standard automated solid-phase synthesis using the Applied Biosystems instrument 430. Following cleavage from the resin the peptide was purified by gel chromatography and preparative HPLC (Vydac C_4 column). Each peptide was characterized by amino acid composition analysis and determined to be greater than 98% pure. ^{125}I-NANP (2000 Ci/mmol) was obtained from Amersham. For the binding studies cells were seeded at 1.5×10^4 cells/cm^2 in Costar 12-well culture dishes. Triplicate wells were used for each experimental point. Cells doubled every 24 h following an initial 1 day lag. Confluent cells (4×10^5 cells/well) were fed on the 4th day with complete medium and used on the 5th. Prior to the addition of peptides, each well was washed 5 × with 1 ml cold binding medium (DMEM/0.1% BSA). Following the addition of ANP peptides, cells were incubated at 4°C in the same medium for the indicated time. Following aspiration of the medium each well was washed 5 times with 1 ml ice-cold binding medium followed by solubilization in 1 ml of 1 N NaOH. Cell-bound radioactivity was determined by gamma counting (LKB) at an efficiency of 78%. Binding data were analyzed using the Ligand PC program [9]. Cells from three representative wells were removed using Trypsin/EDTA and counted in a Coulter counter.

2.3. *Measurement of intracellular cGMP levels*

Each experimental point was determined in triplicate wells. Confluent cultures of cells (see above) in 12-well dishes were washed 5 × with binding medium supplemented with 0.1 mM isobutylmethylxanthine (at 37°C) and incubated for 5 min at 37°C prior to the addition of ANP peptides. Following the addition of the appropriate peptides the cells were incubated at 37°C for 10 min. The medium was then aspirated and 1 ml ice-cold 10% trichloroacetic acid was added to each well. For antagonism studies LANP was added to each well at 500 nM 10 min prior to the addition of NANP. The trichloroacetic acid precipitates were removed by centrifugation and the supernatants extracted extensively with water-saturated ether and dried in vacuo. Following reconstitution and acetylation, the cGMP content of the supernatants was determined in duplicate for each well by cGMP radioimmunoassay (New England Nuclear).

3. RESULTS

To characterize the interactions of ANP with a cultured rat Leydig tumor cell line, ^{125}I-NANP-binding experiments were conducted as described in section 2. All binding experiments described below were performed at 4°C to reduce any possible internalization and/or degradation of the labeled ANP peptide which could result in misinterpretation of the binding data. The association of ^{125}I-NANP (50 pM) with confluent cultures of rat Leydig tumor cells in monolayer was time-dependent with specific binding reaching equilibrium between 90 and 120 min (not shown). Only a small rise in the level of non-specific binding was observed (5% of total binding at equilibrium).

The specific binding of ^{125}I-NANP was saturable while non-specific binding increased linearly with increasing concentration of labeled peptide (fig.1A). Interpretation of the saturation binding data using Scatchard analysis (fig.1A) revealed that ^{125}I-NANP bound to rat Leydig cells with an apparent dissociation constant (K_d) of approx. 0.1 nM. The maximum number of ^{125}I-NANP-binding sites (B_{max}) was calculated to be approx. 30000 per cell. The saturation binding data best fit a one-site model with no significant statistical improvement of the fit obtained by invoking a two-site model.

Competition of ^{125}I-NANP (50 pM) binding by the corresponding non-radioactive peptide is shown in fig.1B. Competition was dose-dependent and 95% complete. Scatchard analysis of these data revealed virtually identical K_d and B_{max} values to those obtained in the saturation binding experiments shown above. Displacement with a 26-amino-acid linear analog of NANP (LANP) is also shown in fig.1B. This analog lacks the 17-membered disulfide ring structure found in the native peptide (see section 2). As shown in fig.1B, this peptide effectively displaced ^{125}I-NANP binding with an estimated competitive inhibition constant (K_i) of approx. 0.16 nM. In addition, Scatchard analysis of the displacement data for LANP revealed a comparable B_{max} value to that obtained for NANP. The same experiment done using ^{125}I-LANP as the tracer ligand yielded results similar to those shown in fig.1B (not shown).

To determine the functional significance of ANP binding to rat Leydig cells, intracellular

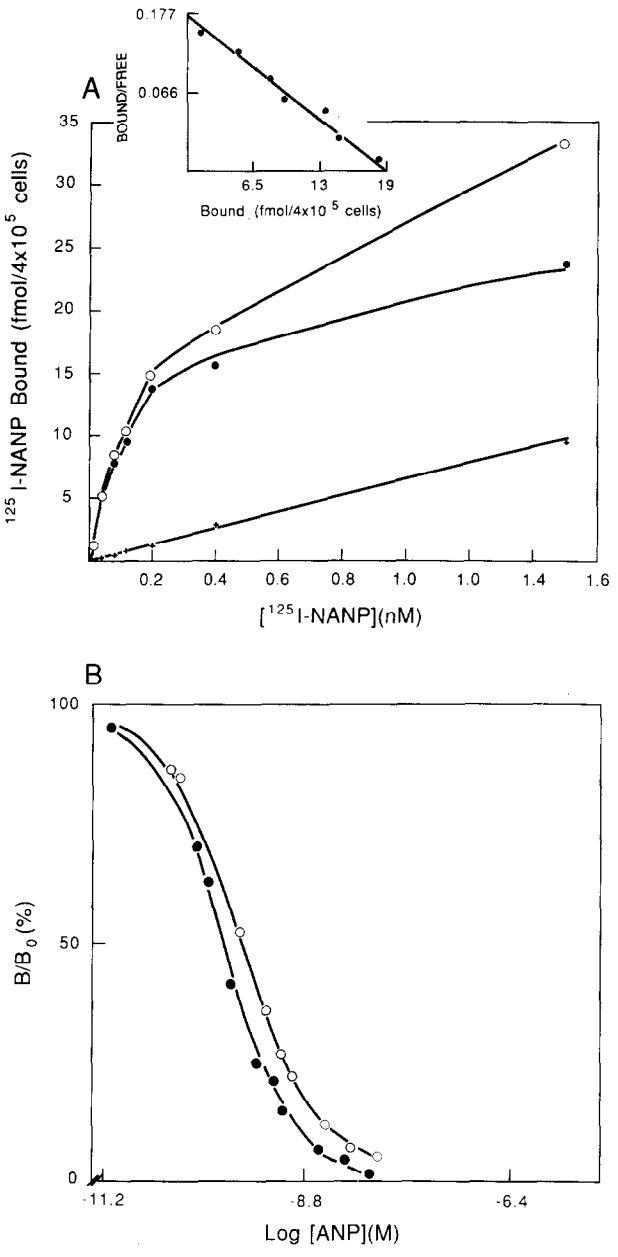


Fig.1. (A) Saturation binding of ^{125}I-NANP to rat Leydig tumor cells. Binding of increasing concentrations of ^{125}I-NANP in the presence (+) and absence (○) of 500 nM non-radioactive NANP at 4°C for 90 min was conducted as described in section 2. Specific binding (●) was calculated as the difference between total and non-specific binding. Each point represents the mean of triplicate determinations from one of two independent experiments. Also shown is the computer-generated Scatchard analysis of the saturation binding data as determined using the Ligand PC program assuming a one-site model [9]. (B) Displacement binding of non-radioactive NANP and LANP to rat Leydig tumor cells. Increasing concentrations of non-radioactive NANP (●) or LANP (○) were incubated in the presence of 50 pM ^{125}I-NANP at 4°C for 90 min and binding determined as described in section 2. Each point represents the mean of triplicate determinations from one of two independent experiments. The solid lines were determined using a non-linear least-square analysis of the binding data.

cGMP levels were measured in response to increasing concentrations of added native and linear ANP as described in section 2. cGMP levels increased in a dose-dependent manner in response to added NANP with an estimated EC_{50} of approx. 8 nM and the maximal response increased 350-fold over basal levels (fig.2). In contrast to NANP, however, LANP did not substantially stimulate cGMP synthesis up to a concentration as great as 500 nM where a slight increase was noted (fig.2). Since LANP bound to rat Leydig cells with a similar affinity to that of NANP (fig.1B), we measured the cGMP response to added NANP in the presence of 500 nM LANP as described in section 2. As shown in fig.2, LANP did not significantly alter the dose dependence or magnitude of the increase in cGMP levels in response to added NANP, suggesting that LANP is not a classical competitive antagonist of NANP in this assay.

4. DISCUSSION

We have demonstrated that a rat Leydig tumor cell line expresses high-affinity receptor-binding sites for ANP. The binding of ANP to these cells was shown to be time dependent and saturable with increasing concentrations of NANP. In addition, the binding of NANP to the rat Leydig tumor cell ANP receptor apparently does not require the intact disulfide-bridge structure found in NANP as demonstrated by the effective competition by LANP on ^{125}I-NANP binding shown in fig.1B.

Another aspect of this work concerns the effect of ANP on the levels of intracellular cGMP in the rat Leydig tumor cell line. As shown in fig.2, NANP elicited a dramatic dose-dependent rise in the levels of intracellular cGMP which was maximally stimulated 350-fold over basal levels. A similar effect of ANP on cGMP levels in normal

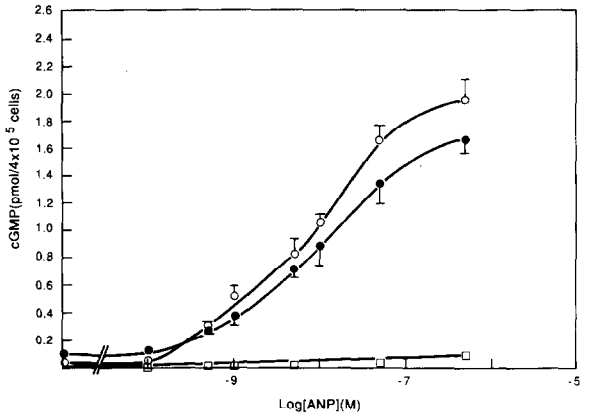


Fig.2. Intracellular cGMP accumulation in cultured rat Leydig tumor cells in response to added ANP peptides. Cells were incubated with increasing concentrations of either NANP (○), LANP (□) or NANP in the presence of 500 nM LANP (●) and intracellular cGMP levels determined as described in section 2. Results represent the mean of triplicate determinations ± SD from two independent experiments.

[10,11] and tumor-derived [12] murine Leydig cells in culture has been reported. In contrast to NANP, the ability of a linear analog (LANP) to stimulate cGMP synthesis in these cells was dramatically attenuated (see fig.2). In the presence of this linear peptide at a concentration of 500 nM (3000-times the K_i determined from the displacement binding studies; fig.1B), the cGMP effect elicited by NANP was not significantly altered (fig.2). This suggests that LANP does not act as a competitive antagonist of NANP in this assay but, instead, appears to be preferentially binding to a predominant high-affinity ANP receptor not coupled to the formation of cGMP. The binding of NANP not only to this 'cGMP uncoupled' form of the ANP receptor but also to a form which results in the formation of cGMP apparently leads to the observed dissociation of high-affinity receptor binding and cGMP formation shown in figs 1A and 2.

The existence of two functional subtypes of the ANP receptor has been demonstrated by other investigators using cultured vascular endothelial [4,5] as well as smooth muscle cells [6]. In addition, it appears that two molecular forms of high-affinity ANP receptors exist in adrenal tissue, one which can catalyze the formation of cGMP and another which cannot [8]. Moreover, a putative function for the cGMP uncoupled form of the ANP receptor has been recently proposed [13]. We have shown that the same phenomenon exists in a cell type not normally associated with the actions of ANP. In addition, we have demonstrated that the 17-membered ring structure found in NANP, although not required for high-affinity binding to the putative cGMP-uncoupled form of the ANP receptor, appears to be extremely important for high-affinity binding and subsequent efficient coupling of the second ANP receptor subtype to guanylate cyclase. These results are not consistent with the report of Pandey et al. [14] which clearly demonstrates a single form of the ANP receptor in a tumor-derived murine Leydig cell line. We believe this is due to the absence of the cGMP-uncoupled form of the ANP receptor in this particular cell line. However, a definitive conclusion awaits a direct comparison of both cell lines using the experimental protocols described above.

Acknowledgements: We would like to thank Dr Mark Polokoff for critically evaluating the manuscript and Carole Hannan for excellent secretarial support.

REFERENCES

[1] Genest, J. and Cantin, M. (1987) Circulation 75, I-118–124.
[2] Jacobs, J.W., Vlasuk, G.P. and Rosenblatt, M. (1987) in: Endocrinology and Metabolism Clinics of North America (Rosenblatt, M. and Jacobs, J.W. eds) vol.16, pp.63–77, Saunders, Philadelphia.
[3] Leitman, D.C. and Murad, F. (1987) in: Endocrinology and Metabolism Clinics of North America (Rosenblatt, M. and Jacobs, J.W. eds) vol.16, pp.79–105, Saunders, Philadelphia.
[4] Leitman, D.C. and Murad, F. (1987) Biochim. Biophys. Acta 885, 74–79.
[5] Leitman, D.C., Andresen, J.W., Kuno, T., Kaminsky, Y., Chang, J.K. and Murad, F. (1986) J. Biol. Chem. 261, 11650–11655.
[6] Scarborough, R.M., Schenk, D.B., McEnroe, G.A., Arfsten, A., Kang, L.L., Schwartz, K. and Lewicki, J.A. (1986) J. Biol. Chem. 261, 12960–12964.
[7] Schenk, D.B., Phelps, M.N., Porter, J.G., Fuller, F., Cordell, B. and Lewicki, J.A. (1987) Proc. Natl. Acad. Sci. USA 84, 1521–1525.
[8] Takayanagi, R., Inagami, I., Snajdar, R.M., Imada, T., Tamura, M. and Misono, K.S. (1987) J. Biol. Chem. 262, 12104–12113.
[7] Waldman, S.A., Rapoport, R.M. and Murad, F. (1984) J. Biol. Chem. 259, 14332–14334.
[8] Winquist, R.J., Faison, E.P., Waldman, S.A., Schwartz, K., Murad, F. and Rapoport, R.M. (1984) Proc. Natl. Acad. Sci. USA 81, 7661–7664.

[9] Munson, P.J. and Rodbard, D. (1984) Computers in Endocrinology, pp.117–145, Raven, New York.
[10] Mukhopadhyay, A.K., Schumacher, M. and Leidenberger, F.A. (1986) Biochem. J. 239, 463–467.
[11] Pandey, K.N., Parlou, S.N., Kovacs, W.J. and Inagami, T. (1986) Biochem. Biophys. Res. Commun. 138, 399–404.
[12] Pandey, K.N., Kovacs, W.J. and Inagami, T. (1985) Biochem. Biophys. Res. Commun. 133, 800–806.
[13] Maack, T., Suzuki, M., Almedia, F.A., Nussenzveiz, D., Scarborough, R.M., McEnroe, G.A. and Lewicki, J.A. (1987) Science 238, 675–678.
[14] Pandey, K.N., Inagami, T. and Misono, K.S. (1986) Biochemistry 25, 8467–8472.