Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 78-85

HIGH AFFINITY RECEPTORS FOR ATRIAL NATRIURETIC FACTOR IN PC12 CELLS

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Received August 1, 1988

Specific receptors for atrial natriuretic factor (ANF) are characterized on PC12 cells (rat pheochromocytoma cell line). Radioiodinated synthetic ANF (Rat, 8-33) bound to a single class of high affinity binding sites with the Kd value of 6.7 X 10^{-10} M. The Bmax value was 29 fmol/10^5 cells and receptor density was calculated as 194,000 ± 20,000/cell. Photoaffinity labelling of ANF receptor specifically labelled two protein bands with apparent m.wt of 70,000 and 130,000. When the cells were incubated with the labelled ligand at 37°C the ligand was internalized. The rate of internalization increased in the presence of increased ligand concentration. ANF receptors on PC12 cells are reported for the first time which would provide a unique model for study of ANF-receptor interaction.

The regulatory role of Atrial Natriuretic Factor (ANF) in fluid volume homeostasis, natriuresis and smooth muscle relaxation (1-2) has been the focus of numerous investigations since the discovery of this new class of peptidic hormone. Specific ANF receptors are localized in various tissues (3) and are found to coexist with guanylate cyclase enzyme activity, a bifunctional protein with a receptor domain for ANF and a catalytic domain for cyclic GMP (4).

The presence of high affinity receptors for ANF on adrenal cortex (5) and adrenal medullary cells (6) suggest that the adrenal glands serve as the target organ for ANF. Inhibition of aldosterone synthesis and release in the adrenal zona glomerulosa (7) and inhibition of catecholamine synthesis in pheochromocytoma tumor cells (8) by ANF confirms the regulatory effect of this hormone on adrenal function, yet the intracellular mechanisms whereby it elicits its control effect(s) is not known. In this study we report the presence of ANF receptors in PC12 cells, a pheochromocytoma cell line which is widely used for the study of

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catecholamines biosynthesis and secretion. This cell line offers a unique model to study the interaction of ANF receptor dynamics with catecholamine synthesis and release.

MATERIALS AND METHODS

Materials

Synthetic rat Atrial Natriuretic Factor (ANF 8-33) and [^{125}I]-ANF (Rat, 8-33, specific activity 1200-1500 Ci/mmole) were purchased from Peninsula Laboratories Inc. (Belmont,CA). Electrophoresis materials were purchased from Bethesda Research Laboratories and *bis*(Sulfosuccinimidyl) suberate was obtained from Pierce Chemical Company. All other chemicals used in these experiments were purity grade.

Cell Culture

PC12 cells were obtained from American Type Culture Collection (CRL NO.1721) and maintained in RPMI medium containing 10% heat inactivated horse serum, 5% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37^{o}C in a humidified atmosphere containing 5% CO2. Just prior to the binding assay, the cells were harvested from tissue culture flasks and washed three times with serum free RPMI medium containing 0.2% BSA.

Binding Experiments

ANF binding assays in intact PC12 cells was performed by incubating 5x10^{5} cells with [^{125}I]-ANF in the binding medium (RPMI medium containing 0.2% BSA) in a total volume of 200 μl, at 0^{o}C for 2 hrs. All assays were performed in polystyrene tubes. At the end of incubation the bound and free radioligand were separated by rapid filtration using GF/C glass fibre filters treated with 0.02% Tween-20 in PBS for 2 hrs. The filters were washed rapidly with an additional 10 ml of ice cold 0.15 M NaCl and counted in a gamma scintillation counter.

Internalization of ANF-receptors

PC12 cells were washed in prewarmed binding medium three times, loosely pelleted by centrifugation at 300 X g and incubated with [^{125}I]-ANF at 37^{o}C. At the end of incubation period the tubes were drawn from the incubator and placed on ice. The cells were washed by adding ice cold 0.15 M NaCl and centrifuged at 300 X g for 2 min. at 0^{o}C. ANF bound to the cell surface was determined by extraction of the intact cells with 0.5 M NaCl, 0.2 M acetic acid (9). The cells were solubilized in 1 N NaOH and the radioactivity associated with the cells was determined which represented the internalized ligand.

Affinity Cross-linking Studies

Confluent cells from 150 cm^{2} flasks were washed with serum free RPMI medium and homogenized in the ice cold binding medium containing 0.25 M sucrose, 0.4 mM phenymethylsulfonyl fluoride, 1.0 mg/ml bacitracin, 20 μg/ml each leupeptin and aprotinin. The homogenate was centrifuged at 1,500 x g for 10 min. and the supernatant was centrifuged at 100,000 x g for 30 min. The pellet was resuspended in the binding medium and centrifuged again at 100,000 x g for 30 min. to remove sucrose. The washing step was repeated twice and the final pellet obtained was incubated with the labelled ligand in a total volume of 200 μl for 90 min at 37^{o}C. 200 μl of 0.2 mM *bis*(sulfosuccinimidyl) suberate in DMSO was added and the incubation was continued for

another 30 min. At the end of incubation the tubes were transferred to ice, cold binding medium was added and centrifuged at 100,000 x g for 30 min. The pellet was solubilized and electrophoresed according to the method of Laemmli (10) using 10% gel. The gels were dried and exposed to Kodak X-OMAT film at -70°C for 30 days.

RESULTS

ANF Receptor Binding

PC12 cells show high affinity, saturable binding for [^{125}I]-ANF. Scatchard analysis yielded a linear plot (fig.1) indicating a single class of binding sites; the apparent dissociation constant (Kd) for ANF was 6.7 X 10^{-10} M and Bmax was 1.94 X 10^{-10} M (29 fmol/10^5 cells). Based on this data the number of binding sites are calculated as 194,000 ± 20,000/cell. Also binding of [^{125}I-ANF was a time dependent process and equilibrium binding was reached within 120 min.(fig.2). 2 μM concentration of cold ANF displaced almost 80% of labelled ligand binding indicating the specificity of receptor binding.

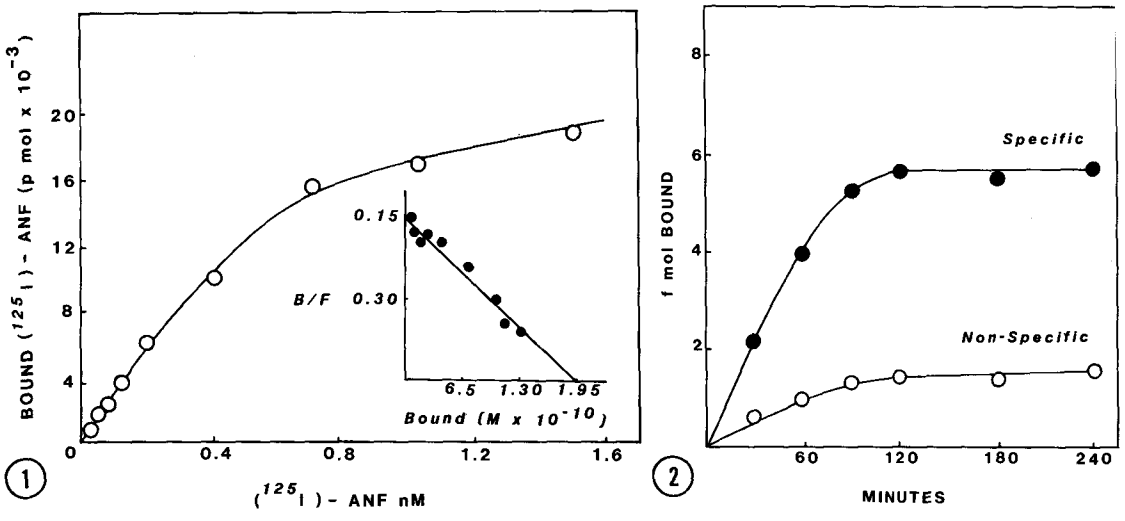

Fig.1: Saturable binding of [^{125}I]-ANF (rat, 8-33) to PC12 cells. The cells (5x10^5 cells/tube) were incubated at 0°C with various concentrations of [^{125}I]-ANF for 2 hrs. in RPMI medium containing 0.2% BSA. Specific binding is the difference between total binding and the non-specific binding in the presence of 2 μM unlabelled ANF (rat,8-33). (Inset) Scatchard plot of the binding data. The calculated Kd is 6.7 x 10^{-10}M and Bmax is 29 fmol/10^5cells.

Fig.2: Binding of [^{125}I]-ANF (rat,8-33) to PC12 cells as a function of time at 0°C with labelled ANF. Non-specific binding was determined in the presence of 2 μM unlabelled ANF (rat, 8-33).

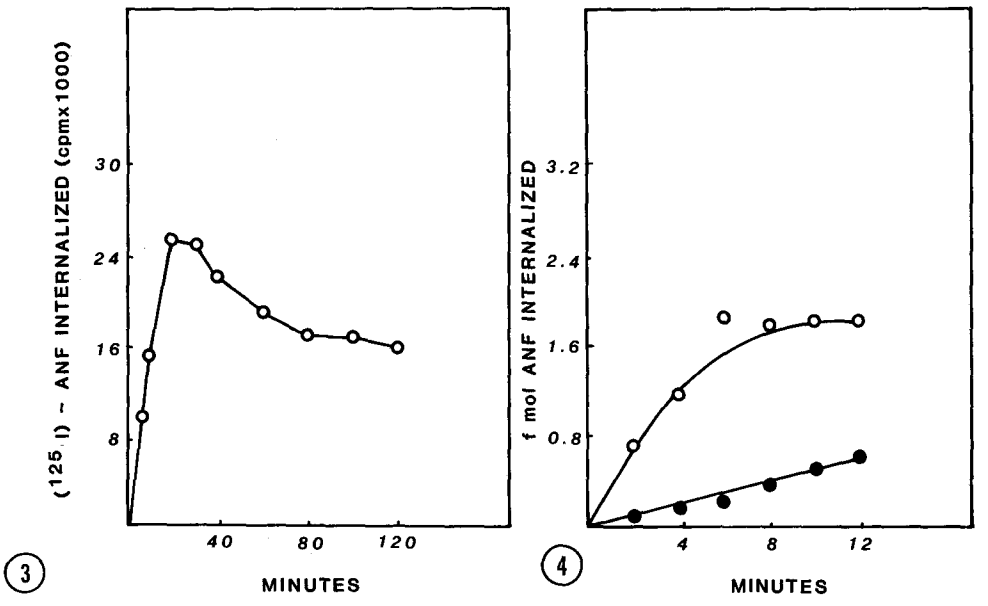

Fig.3: Time course of [^{125}I]-ANF internalization by PC12 cells at 37°C. The cells were incubated for 120 min. with 2 nM [^{125}I]-ANF.

Fig.4: Internalization with 0.25 [●] nM and 2 nM [O] [^{125}I]-ANF concentrations at 37°C.

Internalization of I-125 ANF

When the cells were incubated with [^{125}I]-ANF at 37°C, internalization of ANF was observed. This was indicated by the inability of NaCl/acetic acid to completely remove the cell associated radioactivity. The binding of the ligand was more rapid at 37°C, reaching a maximum at 20 min. (fig.3). When internalization was observed at two different concentrations of [^{125}I]-ANF, the internalization was more rapid at 2 nM ANF as compared to the 0.25 nM concentration (fig.4). The surface binding was also the highest with the 2 nM concentration than the 0.25 nM concentration (fig.5). The endocytic rate constant (Ke) was determined by plotting the ratio of internalized ligand to surface-bound ligand as a function of time of incubation (11). This plot produced a linear curve with a slope of Ke. The calculated Ke for ANF at 0.25 nM concentrations was 0.045 min^{-1} and the endocytic rate constant increased to 0.092 min^{-1} at 2 nM concentrations of ANF (fig.6). The increase in the Ke value at higher concentrations of ANF was concomitant with an increased

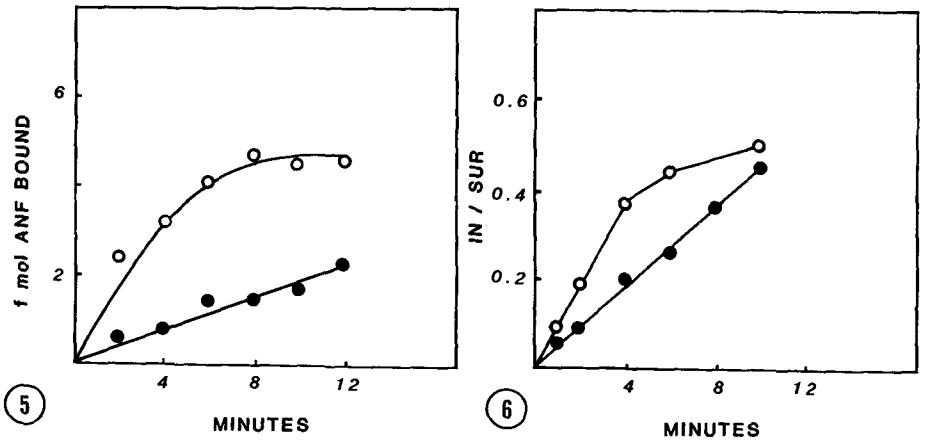

Fig.5: Surface binding of [^{125}I]-ANF on PC12 cells at 37°C with 0.25 nM [●] and 2 nM [O] labelled ANF for different time intervals.

Fig.6: In/Sur analysis in PC12 cells at 37°C with 0.25 nM [●] and 2 nM [O] [^{125}I]-ANF. The ratio of radioactivity associated with the interior to the surface of the cell (In/Sur) was determined. The slope of this curve yielded the endocytic rate constant Ke.

surface binding, thus the observed increase in internalization was apparently due to receptor occupancy.

Affinity Labelling of ANF Receptors

The affinity labelling reaction using *bis*(sulfosuccinimidyl) suberate coupled [^{125}I]-ANF specifically to two protein components of the PC12 cell plasma membrane. The labelled proteins migrated at m.wt of 70,000 and 130,000 on SDS polyacrylamide gel electrophoresis under reducing condition (fig.7). In the presence of 2 μM cold ANF the labelling of both of these proteins was inhibited almost completely, while non-specific labelling of other fractions remained unaltered. This indicated the specificity of ANF binding with 70,000 and 130,000 m.wt proteins in the plasma membrane.

DISCUSSION

A great deal of attention has focused on the influence of ANF on adrenal responses, since the polypeptide inhibits basal aldosterone synthesis and release from the adrenal zona glomerulosa (7). The presence of high affinity receptors for ANF has been reported in adrenal cortex (5), adrenal zona glomerulosa (12), adrenal medullary cells (6) and in human pheochromocytoma cells (8). This suggests that the adrenal glands may be functionally regulated by ANF at various levels. Recent evidence

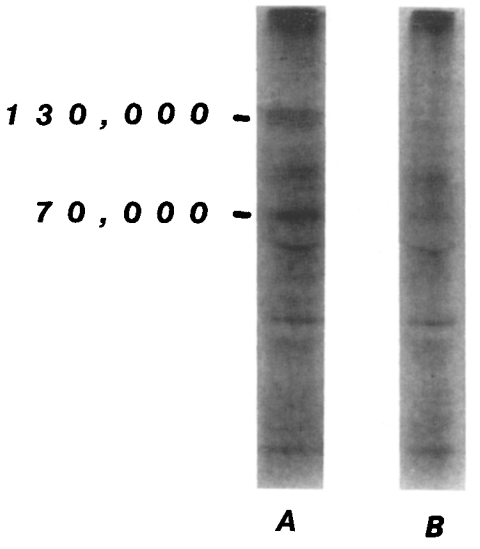

Fig.7: Specificity of [^{125}I]-ANF cross linking to ANF receptors on PC12 cells. The dried gel was exposed to Kodak X-OMAT film at -70°C for 30 days. Specific binding of [^{125}I]-ANF to the receptors in plasma membrane (lane A). Non-specific binding in the presence of 2 μM unlabelled ANF (lane B).

of ANF secretion by adrenal medullary cells (13) indicates that the adrenals may have more complicated roles in mediating the actions of ANF.

The present results demonstrate high affinity receptors for ANF on PC12 cells, a transformed cell line which has served as an interesting model for exploring the functional aspects of chromaffin cells. Binding of ANF to PC12 cells fulfills many of the criteria for specific receptor recognition sites. It was specific, rapid, reversible and saturable. The dissociation constant value (Kd) and Bmax values are comparable to the values reported previously for adrenal medullary (6), renal cortex (14) and cultured vascular smooth muscle cells (15). PC12 cells have been reported to possess high levels of the particulate form of guanylate cyclase which was activated by ANF resulting in the elevation of intracellular cGMP (16). This response is similar to the cGMP elevation observed in rabbit and rat aorta (17), vascular smooth muscle cells (14) and bovine aortic endothelial cells (18). Demonstration of ANF receptors on PC12 cells suggest that the particulate form of guanylate cyclase may be functionally coupled to the ANF receptors, as in many other cells.

Following covalent coupling of radiolabelled ligand to ANF receptors on plasma membranes, two proteins were specifically labelled and migrated at m.wt 70,000 and 130,000 under reducing conditions. These correspond to two classes of receptors reported in bovine adrenocortical cells (5), bovine aortic endothelial cells, aortic smooth muscle cells and human lung fibroblast (19). The high molecular weight protein, isolated from the bovine adrenocortical cells showed both ANF binding and guanylate cyclase activity, which is also believed to exist in reducible and non-reducible forms. The low molecular weight receptors are reported not coupled to particulate guanylate cyclase.

PC12 cells appear to possess two classes of receptors, one linked to the particulate form of guanylate cyclase. Since the high molecular weight receptor was not completely reduced to the low molecular weight protein, as indicated by the presence of two labelled fractions, it is speculated both reducible and non-reducible forms of high molecular weight receptor proteins may be present in PC12 cells. The internalization process is another interesting phenomenon, since the internalization rate varies with the ligand concentration. It is speculated that the internalization process in these cells may perform functions other than receptor-mediated endocytosis, such as transmembrane signal transduction or diminishing the cell responsiveness to the ligand by removing the cell surface receptors (20).

REFERENCES

1. Cantin, M., and Genest, J. (1985) Endocrin. Rev., 6, 107-126.
2. Ingami, T., Misono, K.S., Grammer, R.T., Fukumi, H., Maki, K., Tanaka, I., McKenzie, J.C., Tajayangi, R., Pandey, K.N., and Parmentier, M. (1985) Clin. Exp. Hypertens. Part A theory Pract.,A7, 851-866.
3. Roubert, P., Lonchampt, M.O., Chabrier, P.E., Plas, P., Goulin, J., and Braquet, P. (1987) Biochem. Biophys. Res. Commun., 147, 957-963.
4. Kuno, T., Anderson, J.W., Kamidsaki, Y., Waldman, S.W., Chang, L.Y., Saheki, S., Leitman, D.C., Nakane, M. and Murad, F. (1986) J. Biol. Chem., 261, 5817-5823.
5. Takayanagi, R., Ingami,T., Snajdar, R.M., Imada, T., Tamura,M., and Misono, K.S. (1987) J. Biol. Chem., 262, 12104-12113.
6. Heisler, S. and Morrier, E. (1988) Biochem. Biophys. Res. Commun., 150, 781-787.
7. Goodfriend, T.l., Elliot, M.E., Atlas, S.A. (1984) Life Sci., 35, 1675-1682.

8. Shionoiri, H., Hirawa, N., Takasaki, I., Ishikawa, K., Minamisawa, K., Miyajima, E., Kinoshita, Y., Shimoyama, K., Shimonaka, M., Ishido,M., and Hirose, S. (1987). Biochem. Biophys. Res. Commun., 148, 286-291.
9. Haigler, H.T., Maxfield, F.R., Willingham,M.C., and Pastan, I. (1980) J. Biol. Chem.,255, 1239-1241.
10. Laemmli, U.K. (1970) Nature, 227, 680-685.
11. Wiley, H.S. and Cunningham, D.D. (1982) J. Biol. Chem., 257, 4222-4229.
12. Meloche, S., Ong, H., Cantin, M. and DeLean, A. (1986) J. Biol. Chem., 261, 1525-1528.
13. Pruss, R.M. and Zamir, N. (1987) Neurochem. Int., 11, 299-304.
14. Ogura, T., Mitsui, T., Ogawa, N., Ota, Z. (1985) Res. Commun. Chem. Pathol. Pharmacol., 49, 353-360.
15. Roubert, P., Lonchampt, M.O., Chabrier, P.E., Plas, P., Goulin, J., and Braquet, P. (1987) Biochem. Biophys. Res. Commun., 148, 61-67.
16. Fiscus, R.R., Robles, B.T., Waldman, S.A., Murad, F. (1987) J. Neurochem., 48, 522-528.
17. Rapoport,R.M., Winquist, R.J., Faison, E.P., Waldman, S.A., and Murad, F. (1986) Eur. J. Pharmacol., 120, 123-126.
18. Leitman, D.C. and Murad, F. (1986) Biochem. Biophys. Acta, 885, 74-79.
19. Leitman, D.C., Andresen, J.W., Catalano, R.M., Waldman, S.A., Tuan, J.J. and Murad, F. (1988) J. Biol. Chem., 263, 3720-3728.
20. Minami, Y., Samelson, L.E. and Klausner, R.D. (1987) J. Biol. Chem., 262, 13342-13347.