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SPECIFIC BINDING SITES FOR ATRIAL NATRIURETIC PEPTIDE (ANP) IN CULTURED MESENCHYMAL NONMYOCARDIAL CELLS FROM RAT HEART

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SUMMARY: Specific binding sites for atrial natriuretic peptide (ANP) were studied in cultured mesenchymal nonmyocardial cells (NMC) from rat heart. Binding study using ^{125}I-labeled synthetic rat (r) ANP revealed the presence of a single class of high-affinity binding sites for rANP in cultured NMCs derived from both atria and ventricles; the apparent dissociation constant (K_d) was $\sim$0.2 - 0.3 nM and the number of maximal binding sites was $\sim$190,000 - 300,000 sites/cell. rANP significantly stimulated intracellular cGMP formation of cardiac NMCs in a dose-dependent manner (1.6×10^{-8} M - 3.2×10^{-7} M). rANP had no effect on synthesis of prostaglandin I_2 by cultured cardiac NMCs. The physiological significance of ANP action on cardiac tissue remains to be determined.

Atrial natriuretic peptide (ANP), a potent natriuretic and vasoactive polypeptide recently isolated from mammalian atria (1-7), has been shown to act on kidney to cause natriuresis and diuresis, and on blood vessels to induce smooth muscle relaxation. It has recently been reported that ANP stimulates guanylate cyclase activities in homogenates of several tissues including rat aorta and kidney (8), whereas it is an inhibitor of adenylate cyclase activities from several tissues including rat heart (9), suggesting its potential function(s) in various tissues. In fact, ANP inhibits aldosterone secretion from rat adrenal glomerulosa cells (10).

In the present study, we have attempted to investigate whether there exist ANP receptors in cultured mesenchymal cells from rat heart, and whether it has any effect on formation of

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intracellular cGMP and synthesis of prostaglandin (PG) I_2 by these cells.

MATERIALS AND METHODS

Culture of cardiac mesenchymal cells Cardiac mesenchymal non-myocardial cells (NMC) were obtained by a modified method of enzymatic dissociation (11). Atrial and ventricular tissues excised from adult male Wistar rats were minced and rinsed with Dulbecco's modified Eagle's medium (DMEM). The tissue fragments were dispersed with 0.05% collagenase (Type II; Worthington) and 0.05% trypsin (Difco) at 37°C for 3 successive 30 min intervals. The second and third cell suspensions were combined and centrifuged. The cell pellet was washed with DMEM containing 10% fetal calf serum (FCS; Flow) and antibiotics. The cell suspensions were incubated at 37°C in a humidified atmosphere of 95% air - 5% CO_2 for 4 hrs, during which interval the mesenchymal NMCs quickly attached to the culture dish surface, leaving the myocytes in suspension. Cardiac mesenchymal NMCs thus cultured were readily distinguished by morphological features as previously described (11-13); they were polyhedral with phase-lucent cytoplasma and large and oval nucleus. Cardiac NMCs rapidly proliferated and reached confluency 7 - 10 days after plating. The primary culture was used in the experiments.

Binding experiments Binding studies using ^{125}I-labeled synthetic rat (r) ANP (specific activity: 100 - 120 μCi/μg) were performed as previously reported (14). In brief, the cells were usually incubated with ^{125}I-labeled-rANP in one-ml Hank's medium containing 0.1 % bovine serum albumin (binding medium) at 24°C for 60 min. Specific binding was defined as total binding minus nonspecific binding in the presence of 3.2×10^{-7} M unlabeled rANP.

Determination of intracellular cGMP The cells were incubated with or without rANP in one-ml binding medium containing 0.5 mM methylisobutylxanthine (Calbiochem-Behring) at 37°C for 30 min. Concentrations of intracellular cGMP were determined by radioimmunoassay (RIA) kit (New England Nuclear) after acetylating the samples (14).

Determination of PGI_2 For measurement of PGI_2, the cells were incubated at 37°C for 60 min in one-ml DMEM containing 0.5% FCS in the absence and presence of rANP. After incubation, media were removed and assayed for 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2. Determination of 6-keto-$PGF_{1\alpha}$ was performed by RIA using rabbit anti-6-keto-$PGF_{1\alpha}$ serum (G7BMK6102-7: kindly supplied by Ono Pharmaceutical Co., Osaka, Japan). Cross-reactivity of the antibody with other PGs was as follows: PGE_1 (8%); PGE_2 (9.1%); $PGF_{1\alpha}$ (4.3%); $PGF_{2\alpha}$ (5%). Incubation mixture consisted of 0.1 ml standard or samples, 0.1 ml antibody (final dilution 1:3,500) and 0.1 ml ^{3}H-6-keto-$PGF_{1\alpha}$ (~10,000 cpm: Amersham), all diluted in 0.1 M phosphate buffer, pH 7.4, containing 1 M NaCl and 0.1% gelatin. Incubation was carried out at 37°C for 60 min. Antibody-bound and free ligand was separated by addition of 0.2 ml ice-cold dextran-coated charcoal suspension (0.25% dextran T-70/2.5% Norit A). The minimum detectable amount of 6-keto-$PGF_{1\alpha}$ was 10 pg/tube.

Drugs rANP$^{1-28}$ was purchased from Protein Research Foundation, Osaka, Japan, arachidonate and indomethacin from Sigma Chemical.

RESULTS

Binding of ^{125}I-labeled-rANP to cultured cardiac NMCs was rapid at 24°C, and reached an apparent equilibrium during 30 - 60 min (Fig. 1); specific binding was more than 70% of total binding. An equilibrium binding of ^{125}I-labeled-rANP to cultured NMCs derived from both atria (Fig. 2) and ventricles (Fig. 3) was saturable. Scatchard plots of binding data showed the presence of a single class of high-affinity binding sites for rANP; the apparent dissociation constant (K_d) of rANP to atrial

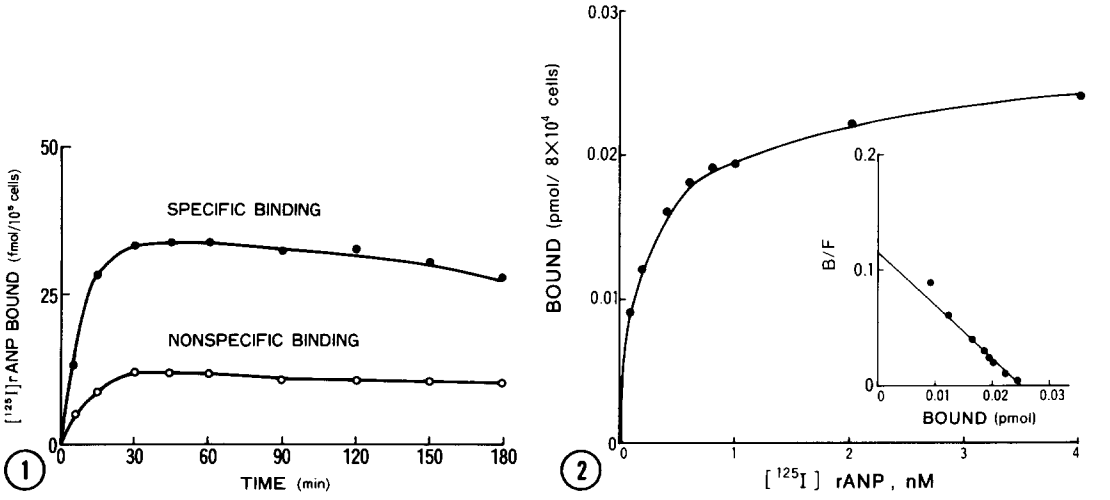

Fig. 1. Binding of ^{125}I-labeled-rANP to cultured rat ventricular nonmyocardial cells as a function of time.
Confluent cells (10^5 cells/well) were incubated at 24°C in one-ml binding medium containing 1 nM ^{125}I-labeled-rANP. Specific binding (●) was obtained by subtracting nonspecific binding (○) in the presence of 3.2×10^{-7} M unlabeled rANP from total binding. Each point is the mean of two experiments.

Fig. 2. Saturable binding of ^{125}I-labeled-rANP to cultured nonmyocardial cells from rat atria.
Confluent cells (8×10^4 cells/well) were incubated at 24°C for 60 min in one-ml binding medium with various concentrations of ^{125}I-labeled-rANP. Specific binding was defined as total binding minus nonspecific binding in the presence of 3.2×10^{-7} M unlabeled rANP. Each point is the mean of two experiments. (Inset) Scatchard plot of binding data. The ratio of bound to free (B/F) ^{125}I-labeled-rANP is plotted against the concentrations of bound radioligand.

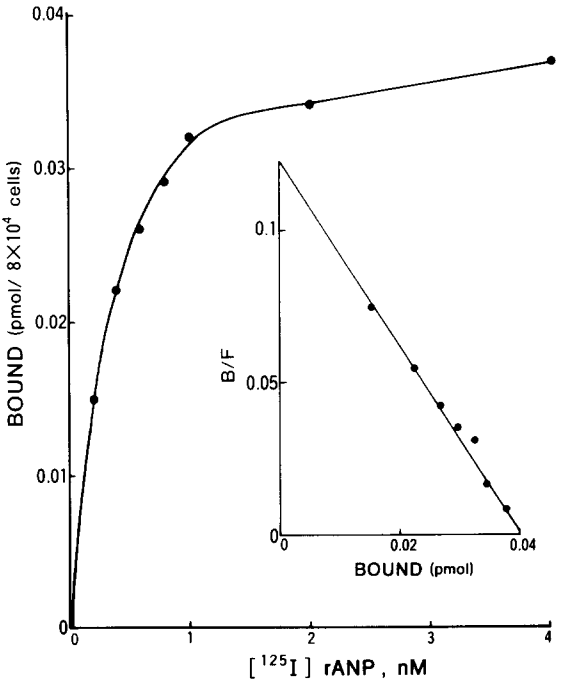

Fig. 3. Saturable binding of ^{125}I-labeled-rANP to cultured non-myocardial cells from rat ventricles.
Confluent cells (8×10^4 cells/well) were incubated with ^{125}I-labeled-rANP in the same manner as in Fig. 2. Each point is the mean of two experiments. (Inset) Scatchard plot of binding data as in Fig. 2.

and ventricular NMCs was 0.23 nM and 0.28 nM, respectively, and the number of maximal binding capacity (B_{max}) was 189,000 sites/cell (atria) and 300,000 sites/cell (ventricles). Unlabeled rANP competitively inhibited the binding of ^{125}I-labeled-rANP to its binding sites in cultured cardiac NMCs (Fig. 4).

rANP stimulated formation of intracellular cGMP in cardiac NMCs from both atria (Fig. 5) and ventricles (data not shown) in a dose-dependent manner: a significant ($p<0.001$) increase was induced with 1.6×10^{-8} M and a maximal stimulation with 3.2×10^{-7} M.

Cultured NMCs from both atria and ventricles produced and secreted into medium comparable amounts of immunoreactive 6-keto-$PGF_{1\alpha}$: atrial NMCs (1.3 ± 0.11 ng/hr/10^5 cells, mean ± SE,

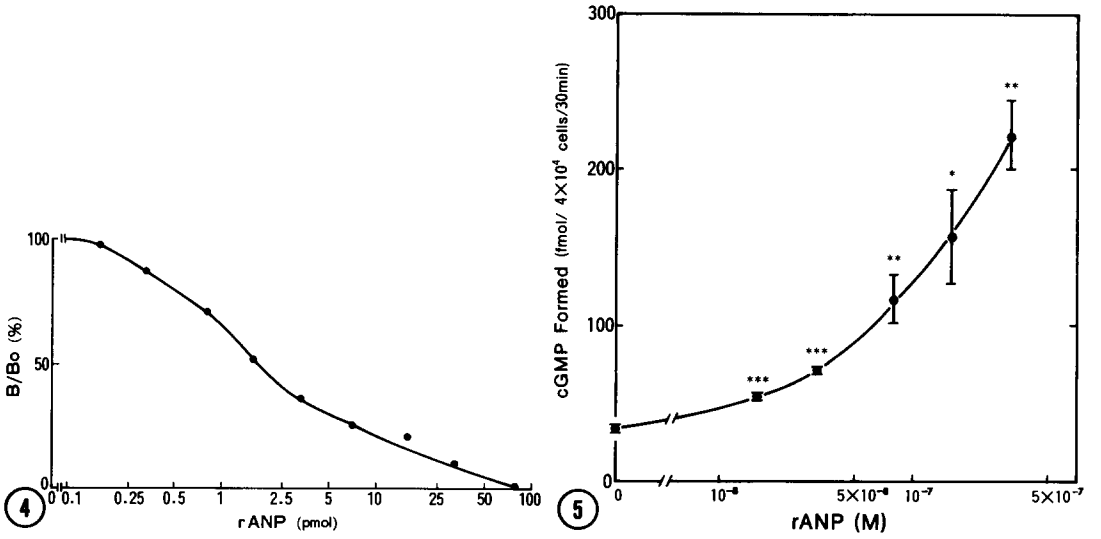

Fig. 4. Competitive binding of ^{125}I-labeled-rANP to cultured rat ventricular nonmyocardial cells by unlabeled rANP.
An equilibrium binding was performed by incubating the cells (10^5 cells/well) at 24°C for 60 min in one-ml binding medium with 1 nM ^{125}I-labeled-rANP in the presence and absence of unlabeled rANP as indicated. Total binding was 8% of ^{125}I-labeled-rANP added, and specific binding was 70% of total binding. Each point is the mean of two determinations.

Fig. 5. Effect of rANP on intracellular cGMP formation in cultured nonmyocardial cells from rat atria.
Subconfluent cells (4×10^4 cells/well) were incubated at 37°C for 30 min in one-ml binding medium containing 0.5 mM methylisobutylxanthine in the absence and presence of rANP in concentrations as indicated. Concentrations of intracellular cGMP were determined by radioimmunoassay after acetylation. Each point is the mean of three samples; bar indicates SEM. Statistically significant from control: (*) $p<0.02$, (**) $p<0.01$, (***) $p<0.001$.

n=6); ventricular NMCs (1.3 ± 0.12 ng/hr/10^5 cells, n=6). rANP ($3.2 \times 10^{-10} - 3.2 \times 10^{-7}$ M) had no effect on synthesis of 6-keto-$PGF_{1\alpha}$ by either atrial or ventricular NMCs (Table 1). In contrast, arachidonate (10^{-4} M) significantly stimulated synthesis of 6-keto-$PGF_{1\alpha}$, whereas indomethacin (10^{-4} M) completely blocked its synthesis, indicating de novo synthesis of PGI_2 by cultured cardiac NMCs (Table 1).

DISCUSSION

Two cell types can be cultured from mammalian heart: cardiac myocytes and mesenchymal NMCs (15). Cardiac mesenchymal

Table 1. Synthesis of 6-keto-$PGF_{1\alpha}$ by cardiac nonmyocardial cells (NMC) in culture

Treatment	Atrial NMC (ng/hr/10^5 cells)	Ventricular NMC (ng/hr/10^5 cells)
None	1.7 ± 0.2	1.4 ± 0.15
rANP 3.2×10^{-10} M	1.5 ± 0.12	1.2 ± 0.22
3.2×10^{-9} M	1.5 ± 0.3	1.5 ± 0.15
3.2×10^{-8} M	1.6 ± 0.12	1.1 ± 0.21
3.2×10^{-7} M	1.3 ± 0.18	1.1 ± 0.12
Arachidonate 10^{-4} M	14 ± 3.2*	15 ± 1.3**
Indomethacin 10^{-4} M	< 0.1	< 0.1

Values are mean ± SEM (n=3).
Asterisks show statistical difference from control:
(*) $p<0.02$, (**) $p<0.001$.

NMCs that quickly attach to the culture dish surface and rapidly proliferate, can be readily separated from cardiac myocytes by the differential attachment technique (11-13). Cardiac NMCs cultured and used in this study appear to be distinct from vascular smooth muscle cells (VSMC) nor endothelial cells because they did not show "hill-and-valley" growth pattern characteristic of VSMC (16), nor "cobble stone" appearance or compact polygonal morphology of endothelial cells (17). The presence of neither actin nor Factor VIII antigen was demonstrated in these cells by immunocytochemical study (unpublished observations). The precise identity of the cardiac NMCs have been unclear and referred to simply as "mesenchymal cells" (12).

The present study first demonstrates that mesenchymal NMCs derived from both atria and ventricles of rat heart have specific receptors for ANP. The characteristics of ANP receptors in cardiac NMCs as demonstrated in this study appear to be comparable to those of VSMCs as reported previously (14); the apparent K_d was 0.2 - 0.3 nM and B_{max} was 190,000 - 300,000 sites/cell. Furthermore, ANP has the same stimulatory effect on intracellu-

lar cGMP formation in cardiac NMCs as in VSMCs (14,18) and endothelial cells (18).

It has been reported that cultured mesenchymal NMCs, but not myocytes, of rat heart synthesize the potent vasodilator PGI_2 (11), and PGs synthesized by heart may affect cardiac function, such as alterations of cardiac contractility and vascular resistance (19,20). Our result of synthesis of 6-keto-$F_{1\alpha}$, a stable metabolite of PGI_2, by cultured cardiac NMCs is consistent with that of previous study (11). In this study, ANP has no effect on synthesis of PGI_2 by cultured cardiac NMCs. We have also observed that ANP has no effect on production of either PGI_2 or PGE_2 by cultured VSMC from rat aorta and endothelial cells from calf pulmonary artery (CPAE), both of which possess specific receptors for ANP (unpublished observations). Therefore, it is suggested that biological action(s) of ANP is not mediated by synthesis of PGs as demonstrated in rat kidney (21).

Although the physiological function(s) of cardiac mesenchymal NMCs is unclear, possible interactions of NMCs with myocytes affecting myocyte differentiation have been postulated (13). Therefore, the physiological significance of ANP action(s) on cardiac tissue remains to be determined. It will also be important to investigate how ANP synthesized by the atrial tissue exerts its effect(s) in cardiac tissue, viz. either locally via paracrine fashion or systemically as a hormone.

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REFERENCES

1. Flynn, T.G., de Bold, M.L., and de Bold, A.J. (1983) Biochem. Biophys. Res. Commun. 117, 859-865.

2. Kangawa, K., and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 118: 131-139.
3. Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.R., Gallupi, G.R., and Needleman, P. (1984) Science 223, 67-79.
4. Seidah, N.G., Lazure, C., Chretien, M., Thibault, G., Garcia, R., Cantin, M., Genest, J., Nutt, R.F., Brady, S.F., Lyle, T.A., Paleveda, W.J., Colton, C.D., Ciccarone, T.M., and Veber, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 2640-1644.
5. Atlas, S.A., Kleinert, H.D., Camargo, M.J., Januszewicz, A., Sealey, J.E., Laragh, J.H., Schilling, J.N., Lewicki, J.A., Johnson, L.K., and Maack, T. (1984) Nature 309, 717-719.
6. Misono, K.S., Fukumi, H., Grammer, R.T., and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 199: 524-529.
7. Kangawa, K., Fukuda, A., Kubota, I., Hayashi, Y., and Matuso, H. (1984) Biochem. Biophys. Res. Commun. 212: 585-591.
8. Waldman, S.A., Rapoport, R.M., and Murad, M. (1984) J. Biol. Chem. 259: 14332-14334.
9. Anand-Srivastava, M.B., Franks, D.J., Cantin, M., and Genest, J. (1984) Biochem. Biophys. Res. Commun. 121: 855-862.
10. Chartier, L., Schiffrin, E., and Thibaut, G. (1984) Biochem. Biophys. Res. Commun. 122: 171-174.
11. Ahumada, G.G., Sobel, B.E., and Needleman, P. (1980) J. Mol. Cell. Cardiol. 12: 685-700.
12. Balk, S.D. (1980) Proc. Natl. Acad. Sci. USA 77: 6606-6610.
13. Simpson, P., and Savion, S. (1982) Cir. Res. 50: 101-116.
14. Hirata, Y., Tomita, M., Yoshimi, H., and Ikeda, M. (1984) Biochem. Biophys. Res. Commun. 125: 562-568.
15. Kasten, F.H. (1972) In Vitro 8: 128-150.
16. Gimbrone, M.A., and Corton , R.S. (1975) Lab. Invest. 33: 16-17.
17. Haudenshild, C.C., Corton, R.S., Gimbrone, M.A., Folkman, J. (1975) J. Ultrastr. Res. 50: 22-32.
18. Schenk, D.B., Johnson, L.K., Schwartz, K., Sista, H., Scarborough, R.M., and Lewicki, J.A. (1985) Biochem. Biophys. Res. Commun. 127: 433-442.
19. January, E.T., and Schottelius, B.A. (1974) Proc. Soc. Exp. Biol. Med. 147: 403-406.
20. Krebs, R., and Schror, K. (1976) Brit. J. Pharmacol. 57: 533-541.
21. Keeler, R. (1982) Can. J. Physiol. Pharmacol. 60: 1078-1082.