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PHYSICAL AND FUNCTIONAL ASSOCIATION OF THE ATRIAL NATRIURETIC PEPTIDE RECEPTOR WITH PARTICULATE GUANYLATE CYCLASE AS DEMONSTRATED USING DETERGENT EXTRACTS OF BOVINE LUNG MEMBRANES

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Coupling of the atrial natriuretic peptide (ANP) receptor to particulate guanylate cyclase has been demonstrated kinetically and chromatographically using bovine lung plasma membranes and their detergent extracts. Addition of ANP to the membrane suspension stimulated guanylate cyclase activity 2-5-fold indicating the presence of ANP-sensitive particulate guanylate cyclase. The enzyme retained the ability to respond to ANP even after solubilization with digitonin. Characterization of the solubilized enzyme by gel filtration and affinity chromatography revealed that the ANP receptor and particulate guanylate cyclase exist as a functionally but not covalently linked stable complex.

Atrial natriuretic peptide(ANP), a 28-amino acid peptide hormone released from atria(1-11), appears to cause vascular smooth muscle relaxation and renal excretion of sodium and water by selectively activating, at its target tissues, particulate guanylate cyclase (G-cyclase), the enzyme catalyzing the formation of cGMP from GTP(12-16). Intramembranous interactions between the ANP receptor and G-cyclase are, therefore, the key processes in transducing the information associated with receptor occupancy into the appropriate cellular responses mediated by the second messenger cGMP.

While attempting to isolate ANP receptor and membrane-bound G-cyclase and reconstitute them into a functionally linked complex, we noticed that their coupling is tight enough to survive mild detergent solubilization. We report here the solubilization and properties of ANP-sensitive G-cyclase from the bovine lung with digitonin. Gel filtration and affinity chromatography of the extract revealed the presence of 1:1 complex, in addition to the free forms, of ANP receptor and G-cyclase. The ratio of

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free to complexed forms varied depending on the extraction conditions. The results indicate that the receptor and the effector enzyme are physically separable entities, eliminating the possibility recently suggested by Kuno *et al.*(17) that both ANP-binding and G-cyclase activities reside in the same molecule.

MATERIALS AND METHODS

Affi-Gel 10 was obtained from Bio-Rad; rat ANP(1-28) was from Peptide Institute, Osaka, Japan; ^{125}I-ANP(2000 Ci / mmol) was from Amersham; creatine phosphokinase was from Sigma; digitonin was from Wako Pure Chemicals, Osaka, Japan; cGMP assay kits were from Yamasa Shoyu, Chiba, Japan; Superose 6 was from Pharmacia; fresh bovine lungs were from Shibaurazoki, Tokyo.

Membrane Preparation and Detergent Solubilization—After dissection, bovine lung was homogenized in 5 volumes of ice-cold 1 mM $NaHCO_3$ (pH8.3) containing 5 mM EDTA, 0.2 mM PMSF, 5 μg / ml of pepstatin A and 5 μg / ml of leupeptin with a Waring blender. The homogenate was centrifuged at 8,000 x *g* for 30 min. The pellet was resuspended in the original volume of the same buffer and centrifuged again. This washing was repeated 4 times followed by a wash with 20 mM phosphate-buffered saline (pH7.4). The washed pellet was freeze-thawed and suspended in 50 mM Tris-HCl, pH7.6, containing 10 mM theophylline and 0.1 mM dithiothreitol (DTT) with a Polytron homogenizer at a medium setting, and centrifuged. After two washes, the final pellet (referred to as particulate fractions) was suspended in the Tris buffer containing theophylline and DTT, and extracted by adding digitonin to a concentration of 0.5%. The mixture was stirred for 2 h at 4°C and centrifuged at 100,000 x *g* for 1 h to obtain clear digitonin extracts.

Guanylate Cyclase Activity—Digitonin extracts and particulate samples were incubated with GTP-Mn as substrate at 37°C for 15 min according to Kimura and Murad(18), and the reaction mixtures were succinylated and radioimmunoassayed for cGMP.

Gel Filtration-Digitonin extracts (200 μl) were chromatographed on a Superose 6 column (1 x 30 cm) using a Pharmacia FPLC system. The column was developed with 50 mM Tris-HCl, pH7.6, containing 0.1% digitonin at a flow rate of 0.4 ml / min. Fractions of 0.3 ml were collected and assayed for G-cyclase and ^{125}I-ANP-binding activities.

Affinity Chromatography-ANP (0.5 mg) was coupled to Affi-Gel 10 (0.5ml) according to the supplier's instruction. After washing, the gel was poured into a column and equilibrated with 50mM Tris-HCl, pH 7.6, containing 0.1% digitonin. Samples(20 ml)were applied to the column at a rate of 10 ml/h and room temperature, and extensively washed with the equilibration buffer. After confirming that ANP receptors were completely retained by the affinity gel, G-cyclase activities bound to the column were measured to estimate the amount of G-cyclase ANP receptor·complexes present in the samples (digitonin extracts).

Other methods—Specific binding of ^{125}I-ANP to its solubilized receptor was measured as described previously(19). Protein concentration was determined by the method of Bensadoun *et al.*(20).

RESULTS AND DISCUSSION

Solubilization of ANP-sensitive Guanylate Cyclase—Plasma membranes were prepared from bovine lung and extensively washed to remove

contamination with soluble G-cyclase which is biochemically and immunologically distinct from the particulate form of the enzyme(21-23). When G-cyclase activity in the membrane preparations was assayed in the presence of 10^{-7} M ANP, a maximal activation of 2-5-fold was obtained compared to untreated controls. Similar results were also reported in other target tissues such as aorta, kidney, and adrenal by Murad and his associates(12,13,15).

We next examined the effects of ANP on detergent-solubilized particulate G-cyclase. As shown in Fig.1A, ANP stimulated the activity of digitonin-solubilized G-cyclase in a concentration-dependent manner; the threshold concentration of ANP for causing cGMP rise was 0.5 nM and the EC_{50} 5 nM; at 10^{-7} M, it enhanced the G-cyclase activity 1.8-fold. Fig. 1B illustrates the time course of the ANP stimulation of digitonin-solubilized G-cyclase activity. The stimulatory effect was maintained only for 15 min, after which the stimulation decreased. The mechanism of this leveling off of activity at longer times is not clear but could be due to differences in stability between the activated and resting states of the enzyme.

The above results suggest that the particulate G-cyclase and the ANP receptor exist as a stable complex and remain associated even after solubilization with a mild detergent.

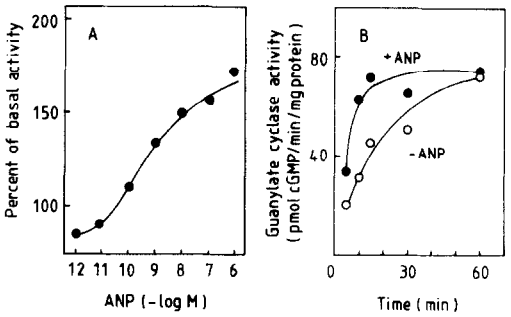

Fig. 1. *A*, Dose-dependent activation by ANP of guanylate cyclase in digitonin extracts. Digitonin extracts from bovine lung were assayed for guanylate cyclase activity in the presence of the indicated concentration of ANP. Following a 15-min incubation, the reaction product cGMP was quantitated by radioimmunoassay. Results were represented as a percentage of the value obtained without ANP under the same conditions which was 27.4 pmol cGMP/min/mg protein. *B*, Time course of ANP-stimulation of guanylate cyclase activity in digitonin extracts.

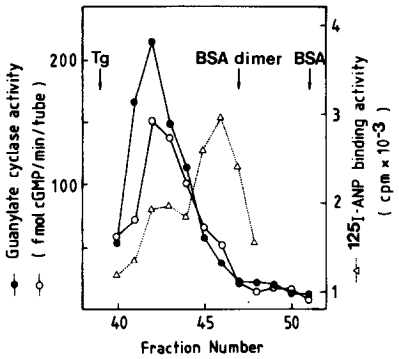

Fig. 2. Gel filtration of digitonin extracts on a Superose 6 column (1.0 x 30 cm). Fractions were assayed for ^{125}I-ANP-binding activity (--△--) and guanylate cyclase activity in the absence (—○—) or presence (—●—) of 10^{-7}M ANP. The size markers used were: thyroglobulin (M_r=669 kDa), bovine serum albumin dimer (M_r=134 kDa), and bovine serum albumin(M_r=67 kDa).

ANP Receptor and Particulate Guanylate Cyclase Are Independent Proteins That Are Closely Coupled—When the digitonin extracts of bovine lung were separated on an FPLC column of Superose 6, two peaks of ANP receptor activity were observed(Fig. 2). The apparent molecular sizes of the two peaks were 320 ± 40 kDa and 150 ± 25 kDa. The former peak co-eluted with G-cyclase activity and the addition of ANP (10^{-7}M) to this fraction caused a significant activation of the G-cyclase (Fig. 2). This result suggests the presence of 1:1 complexes of ANP receptor and particulate G-cyclase in the 320 kDa fraction since both the receptor(19) and particulate G-cyclase(17,24,25) have a similar M_r of about 140,000. The ratio of 320 kDa to 150 kDa form of the receptor varied (5-20%) depending on the conditions such as detergent concentration and length of extraction period.

Affinity chromatography of the extract on ANP-Affi-Gel 10 revealed two populations of G-cyclase: one bound to the affinity gel and the other passed through the column, constituting 5-10 and 90-95% of total particulate G-cyclase activity, respectively (Table I). These two species may represent G-cyclase complexed with ANP receptor through which it was absorbed to the gel and free G-cyclase. The free G-cyclase in the flow-through fractions seems to exist as a dimer since it migrated with an

Table I. Separation of G-cyclase complexed with ANP receptor from free G-cyclase by affinity chromatography to show that certain amounts of particulate G-cyclase exist as a stable complex with ANP receptor. Note the marked difference between the values indicated by *asterisks*

Fractions		ANP receptor[a] (fmol/mg protein)	G-cyclase[b] (%)
Extract		88.3	100
Control Gel	unbound	88.1	102
	bound	ND	< 0.001*
ANP-Affi-Gel 10	unbound	< 0.1	97
	bound	ND	8*

a) Calculated from the Scatchard analysis(27).
b) Represented as a percentage of the value compared with G-cyclase activity in digitonin extract which was 222 pmol of cGMP/min/mg of protein.
ND: not determined.

M_r=300,000 when chromatographed on Superose 6. As expected, virtually no ANP receptor activity was detected in the flow-through fractions.

Whether the free forms of ANP receptor (revealed by gel filtration) and G-cyclase(revealed by affinity chromatography) represent the components dissociated from the ANP receptor·enzyme complexes during extraction remains to be determined. It would be also interesting to determine whether the heterodimeric(a form associated with ANP receptor) to homodimeric transition of the particulate G-cyclase occurs *in vivo* and plays a role in the signal transduction. Very recently Tremblay *et al.*(16) have reported solubilization of ANP-sensitive particulate G-cyclase from bovine adrenal cortex with Triton X-100; Eldeib *et al.*(26) have demonstrated co-solubilization of G-cyclase with heat-stable enterotoxin receptors from intestinal brush-border membranes using digitonin. Although they did not characterize the solubilized receptor-effector systems, their preparations also seem to contain the directly coupled receptor·G-cyclase complexes.

In the present communication, we demonstrated that the ANP receptor ·G-cyclase complex can be solubilized in a functionally coupled state. The coupling seems to be tight since, in other transmembrane signaling systems, solubilization of receptors usually results in the disruption of interaction with other proteins. The availability of ANP-sensitive

particulate G-cyclase in digitonin micelles would allow detailed analysis of the receptor-effector interactions using, for example, fluorescent or spin-labeled probes, which in turn lead to better understanding of the mechanism of G-cyclase activation by ANP.

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REFERENCES

1. Sagnella, G.A., and MacGregor, G.A. (1984) Nature **309**, 666-667
2. Palluk, R., Gaida, W., and Hoefke, W. (1985) Life Sci. **36**, 1415-1425
3. De Bold, A.J. (1985) Science **230**, 767-770
4. Cantin, M., and Genest, J. (1985) Endocrine Rev. **6**, 107-127
5. Maack, T., Camargo, M.J.F., Kleinert, H.D., Laragh, J.H., and Atlas, S.A. (1985) Kidney Int. **27**, 607-615
6. Winquist, R.J. (1985) Life Sci.**37**, 1081-1087
7. Sonnenberg, H. (1985) Klin. Wocheneschr. **63**, 886-890
8. Blaine, E.H. (1985) Clin. Exper. Hypertens. **A7**, 839-850
9. Laragh, J.H. (1985) N. Eng. J. Med. **313**, 1330-1340
10. Needleman, P., and Greenwald, J.E. (1986) N. Eng. J. Med. **314**, 828-834
11. AcKerman, U. (1986) Clin. Chem. **32**, 241-247
12. Waldman, S.A., Rapoport, R.M., and Murad, F. (1984) J. Biol. Chem. **259**, 14332-14334
13. Winquist, R., Faison, E.P., Waldman, S.A., Schwarts, K., Murad, F., and Rapoport, R.M. (1984) Proc. Natl. Acad. Sci. USA **81**, 7661-7664
14. Tremblay, J., Gerzer, R., Vinay, P., Pang, S.C., Beliveau, R., and Hamet, P. (1985) FEBS Lett. **181**, 17-22
15. Waldman, S.A., Rapoport, R.M., Fiscus, R.R., and Murad, F. (1985) Biochim. Biophys. Acta **845**, 298-303
16. Tremblay, J., Gerzer, R., Pang, S.C., Cantin, M., Genest, J., and Hamet, P. (1986) FEBS Lett. **194**, 210-214
17. Kuno, T., Andresin, J.W., Kamisaki, Y., Waldman, S.A., Chang, L.Y., Saheki, S., Leitman, D.C., Nakane, M., and Murad, F. (1986) J. Biol. Chem. **261**, 5817-5823
18. Kimura, H., and Murad, F. (1974) J. Biol. Chem. **249**, 6910-6916
19. Hirose, S., Akiyama, F., Shinjo, M., Ohno, H., and Murakami, K. (1985) Biochem. Biophys. Res. Commun. **130**, 574-579
20. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem **70**, 241-250
21. Garbers, D.L. (1978) J. Biol. Chem. **253**, 1898-1901
22. Hafuenauer-Tsapis, R., Ben Salah, A., Lacombe, M.-L., and Hanoune, J. (1981) J. Biol. Chem. **256**, 1651-1655
23. Nakane, M., and Deguchi, T. (1982) FEBS Lett. **140**, 89-92
24. Radany, E.W., Gerzer, R., and Garbers, D.L. (1983) J. Biol. Chem. **258**, 8346-8351
25. Salah, A.B., Eberentz-Lhommw, C., Lacombe, M., and Hanoune, J. (1983) J. Biol. Chem. **258**, 887-893
26. Eldeib, M.M.R., Parker, C.D., Veum, T.L., Zinn, G.M., and White, A.A. (1986) Arch. Biochem. Biophys. **132**, 51-65
27. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. **51**, 660-672