Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 82-88

ATRIAL NATRIURETIC POLYPEPTIDES (ANP): RAT ATRIA STORE HIGH MOLECULAR WEIGHT PRECURSOR BUT SECRETE PROCESSED PEPTIDES OF 25-35 AMINO ACIDS

Olli Vuolteenaho, Olli Arjamaa and Nicholas Ling*

Department of Physiology, University of Oulu, 90220 Oulu, Finland
*Laboratories for Neuroendocrinology,
The Salk Institute for Biological Studies,
10010 North Torrey Pines Road, La Jolla, California, 92037

Received April 18, 1985

SUMMARY. A radioimmunoassay was developed for rat atrial natriuretic polypeptides. Using the radioimmunoassay and gel filtration in reducing and dissociating conditions, extracts of rat atria were found to contain mainly a 15000-dalton immunoreactive material, probably corresponding to pronatriodilatin. However, when isolated beating atria were incubated in plasma-free conditions, the secreted immunoreactive material consisted almost exclusively of 2500-3500-dalton peptide(s). These results show that rat atrial cells secrete a low molecular weight natriuretic peptide which is highly active, but store the less active large molecular weight form(s).

The heart atria contain potent natriuretic, diuretic and vasoactive polypeptides (atrial natriuretic polypeptides, ANPs) (1). All the peptides are derived from a 126-amino acid atrial protein called pronatriodilatin (2), atriopeptigen (3) or gamma-ANP (4). A large number of homologous ANPs have been isolated from rat and human atria (4-11). It is not known which of these is responsible for the biological activity observed in intact animals. It appears that the full biological activity of the ANPs requires the 25 C-terminal residues of pronatriodilatin and does not tolerate long N-terminal extensions of this fragment (7, 12). Therefore, it is surprising that rat and human atria have been reported to contain a very large amount of pronatriodilatin, which clearly has lower biological activity than the C-terminal fragments (4,7). We report here that rat atria indeed store mainly the large molecular weight ANP, pronatriodilatin, but secrete almost exclusively processed ANP with a molecular weight of 2500-3500 daltons.

ABBREVIATIONS: ANP: atrial natriuretic polypeptide, RIA: radioimmunoassay.

0006-291X/85 $1.50

MATERIALS AND METHODS

Preparation of antisera to ANP.

Synthetic rat ANP (the 26 C-terminal residue fragment of rat pronatriodilatin, H-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH) was prepared by solid phase methodology as in (13). The peptide was conjugated to bovine thyroglobulin with water-soluble carbodiimide as described previously for beta-endorphin (14). The conjugate was emulsified in an equal volume of Freund's complete adjuvant and applied subcutaneously at multiple sites on the backs of five rabbits (0.3 mg of the conjugate in 2 ml per rabbit).

Radioiodination of ANP.

^{125}I-ANP was prepared by the chloramine-T technique (15) and purified by reverse-phase extraction with Sep-Pak C18 cartridges (16). The iodinated peptide was recovered from the cartridge with 3 ml of 50% 2-propanol in 0.1 M acetic acid. The specific activity of the tracer prepared this way was approximately 150 mCi/mg. the iodinated peptide could be stored for at least a month at +4°C.

Radioimmunoassay procedure.

The radioimmunoassays (RIA) were performed in Minisorp (NUNC) polyethylene tubes. The RIA buffer was 0.1 M sodium phosphate, 0.05 M NaCl, 0.1% Triton X-100, 0.02% sodium azide and 0.3% human gamma-globulin, pH 6.0. Standards, controls and unknowns were pipetted in 0.1 ml in duplicate followed by 0.1 ml of the diluted antiserum and 10000 cpm of ^{125}I-ANP. Incubation was at +4°C overnight. The bound and free antigens were separated with 1 ml of 20% polyethylene glycol 6000 (Fluka) in 0.05 M disodium phosphate followed by centrifugation (4500 g, 25 min). The radioactivity in the precipitates was counted in a LKB-Wallac 12-channel gamma-counter.

Extraction of tissue.

Heart atria (the combined block of atria and auricles) were excised immediately after decapitation from male Sprague-Dawley rats (400 g). They were snap-frozen and stored at -20°C or used for the incubations (see below). The atria were homogenized with an Ultra-Turrax in 9 volumes of boiling 1 M acetic acid, containing 0.5% 2-mercaptoethanol, 10 mg/l phenylmethylsulfonyl fluoride, 1 mg/l pepstatin-A (Sigma). The extracts were kept in a boiling water bath for 10 min, then cooled and centrifuged (1000 g, 30 min, +4°C). The supernatants were stored at -20°C. The recovery of added radioiodinated ANP was >90%. On some occasions fresh atria were homogenized in 9 volumes of boiling 4 M guanidine-HCl, 0.02% bovine serum albumin, 0.5% 2-mercaptoethanol (17).

Incubation of isolated rat atria.

Beating atria were incubated in physiological salt solution (154 mM NaCl, 5.6 mM KCl, 5.0 mM Hepes, 2.2 mM $CaCl_2$, 0.12 mM $MgCl_2$ and 2.5 mM glucose, pH 7.4) at 34° with continuous gassing (100% O_2). Aliquots of 0.25 ml of the incubation medium (30 ml total volume) were removed at 5 min intervals for ANP RIA. At the end of the incubation (35-60 min) the medium was lyophilized and the atrial fragment was snap-frozen and stored at -20°C. The atria were viable during the whole incubation period as judged from the regular beating and they remained functional for at least four hours in these conditions.

Gel filtration.

Gel filtration of the atrial extracts and the incubation media was performed in Sephadex G50SF columns (0.7 X 46 cm). Elution was performed with 50% acetic acid, 0.02% 2-mercaptoethanol at +4°C. The flow rate was 2.5 ml/h and fractions of 0.5 ml were collected. The columns were loaded with 0.2 mg-equivalent of the atrial extracts or a lyophilizate from 5 ml equivalent of the incubation media in 0.2-0.5 ml 50% acetic acid and 0.5% 2-mercaptoethanol. Aliquots of the collected fractions were dried in a Savant Speed-Vac with 0.1 mg bovine serum albumin for use in RIA. Trace quantities of Blue Dextran (Pharmacia), radioiodinated ANP and NaCl were included in the samples as internal standards. Recovery of radioactivity and immunoreactivity was 80-105%. In addition, the columns were calibrated with human beta-lipotropin (18), (M_r 11700), human beta-endorphin (13), (M_r 3500) and beta-endorphin (1-18) (18), (M_r 2000). Guanidine-HCl extracts of fresh atria were chromatographed in BioGel P10 columns (0.7 X 46 cm) eluted with 4 M guanidine-HCl as described by Rossier et al. (17). This type of chromatography was not used for the incubation medium because of the extensive dilution required for RIA.

RESULTS

A sensitive RIA for rat ANP was developed in order to study the secretion of ANP. Antiserum designated 7-44-4-1-85 at a final dilution of 1/35000 was used in the present study. The RIA had a sensitivity of 5 pg/tube and the usable range was between 5 and 500 pg/tube (Fig. 1). Intra- and inter-assay coefficients of variation were always <10% and <15%, respectively, at 10 pg to 250 pg ANP/tube. Rat atrial extracts and the medium from incubation of isolated atria gave displacement curves parallel to that produced by synthetic rat ANP (Fig. 1). Using this RIA, acetic acid extracts of rat atria were found to

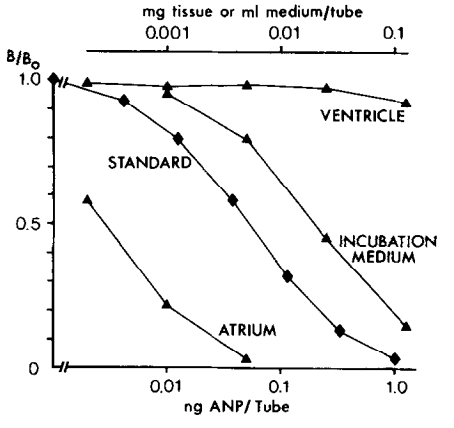

Figure 1. Dose-response curves showing the displacement of the I^{125}-ANP from binding to the specific ANP antibody by the synthetic rat ANP standard, heart atrial and ventricular extracts and lyophilized medium from the incubation of isolated rat atria.

contain 100-300 ng ANP-equivalents of immunoreactivity/mg tissue (n=10). Rat heart ventricles did not contain any detectable amounts of immunoreactivity (Fig. 1), demonstrating the specificity of the RIA. Similar recovery of immunoreactivity was obtained for the guanidine-HCl extracts. Sephadex G50SF gel filtration of the acetic acid extract from either native or incubated atria in reducing and dissociating conditions showed that most of the immunoreactivity corresponds to a high molecular weight material eluting at the void volume (Fig. 2, upper panel). Only about 5-10% of the material was of a smaller molecular weight. The exclusion limit of the gel under these conditions is about 10000 daltons. The same elution pattern was obtained when guanidine-HCl/2-mercaptoethanol extracts of rat atria were chromatographed in BioGel P60 with guanidine-HCl as the solution medium (data not shown). The BioGel chromatography suggested a M_r of 15000 for the principal immunoreactive material from the atrial extract.

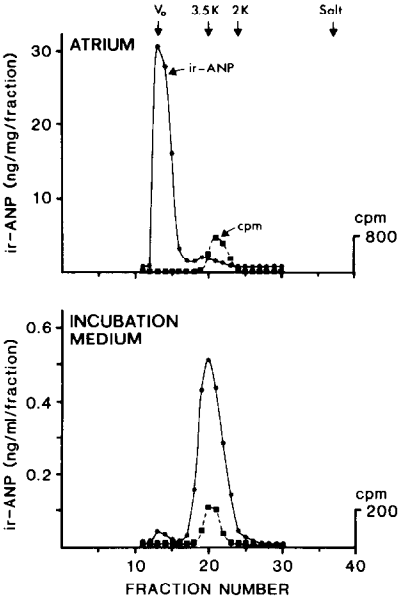

Figure 2. Gel filtration in Sephadex G-50SF of an extract from rat atria (upper panel) or lyophilized medium (lower panel) obtained from a 45-minute incubation of the isolated, beating atrial fragment. The column was eluted at +4°C with 50% acetic acid and 0.02% 2-mercaptoethanol at a flow rate of 2.5 ml/h. Fraction size 0.5 ml. The arrows show the elution positions of human beta-endorphin (3.5K) and beta-endorphin(1-18) (2K). Human beta-lipotropin elutes in the void volume. The elution of radioiodinated ANP, used as an internal standard, is also shown (cpm).

Incubation of isolated atria resulted in the release of immunoreactive ANP into the incubation medium and the rate of release was linear for at least up to one hour. The secretion rate was 74 ± 24 ng/h/fragment (n=7), which corresponds to about 0.25% of the total amount of ANP-like immunoreactivity present in an atrial fragment. When lyophilized aliquots of the media were chromatographed in Sephadex G50SF in reducing and dissociating conditions, a completely different elution pattern was obtained in comparison with that from atrial extracts: most of the secreted immunoreactive material eluted at the 2500-3500-dalton region with only a very small peak at the void volume (Fig. 2, lower panel). The chromatography was repeated ten times with seven samples from atrial extracts and three samples from incubation media. Identical results were obtained among all the atrial extracts. The same finding also held for the incubation media.

DISCUSSION

The present results show that there is a difference between the molecular forms of rat atrial ANP that is being stored versus that which is being secreted. Our experimental design eliminated the possibility that the processing of secreted pronatriodilatin could have taken place in plasma or in the target cells. The atria appear to store huge amounts of a 15000-dalton pronatriodilatin-like material, which is reported to be biologically less active than the smaller ANPs (7,12). The secreted ANP, on the other hand, has an apparent molecular weight of 2500-3500 daltons corresponding to peptides with about 20-35 amino acids. However, since the full biological activity of ANP requires the 25 C-terminal residues of pronatriodilatin, it seems reasonable to propose that the secreted ANP(s) contains at least these residues. Although the secreted ANP elutes in gel filtration as a symmetrical peak, the broadness of the peak suggest that it could consist of multiple components (see Fig. 2). Our antiserum is directed towards the C-terminus of pronatriodilatin and, although its cross-reactivity with the larger molecular forms are not known, it probably recognizes with similar avidity all ANPs containing the 101-126 sequence of pronatriodilatin, because the antigen used was the synthetic rat

pronatriodilatin (101-126). In a recent paper, Nakao et al (20) have also shown that the major molecular form of ANP from rat atrial extract is the high molecular weight gamma-ANP.

At present we do not have an explanation for the seemingly paradoxical finding that the rat atria store mainly a relatively inactive form of ANP, namely pronatriodilatin (4,7), but secrete a much lower molecular weight but biologically active peptide. It seems that either the processing of pronatriodilatin to the more active peptides is closely connected with the secretion process or that the atrial cells have the capability of secreting selectively the small proportion of processed ANP which is present in the atrial extract (see Fig. 2). In another paper we have shown by immunohistochemical method that most of the ANP containing granules in rat atria are located in the sarcoplasmic core near the nuclei of the atrial cells and that only a very small proportion of the granules reside near the plasma membrane (19). It is tempting to speculate that pronatriodilatin maturates to the secreted form(s) during the transport of the granules from the sarcoplasmic core to the plasma membrane. In this way the selective secretion of the processed form(s) could easily be achieved. In any case our novel findings show that the processing of pronatriodilatin takes place in the atrial cells and that it follows a rather unusual course. In future studies it would be very important to study the exact mechanism of the processing and determine the precise molecular nature of the secreted ANP(s).

ACKNOWLEDGEMENTS

We thank Mrs. Eila Nimmo for typing the manuscript and Mrs. Anneli Rautio for the drawings.

REFERENCES

1. Saquella, G.A. and MacGregor, G.A. (1984) Nature 309, 666-667.
2. Lazure, C., Seidah, N.G., Chretien, M., Thibault, G., Garcia, R., Cantin, M. and Genest, J. (1984) FEBS Lett. 172, 80-86.
3. Geller, D.M., Currie, M.G., Siegel, N.R., Fok, K.F., Adams, S.P. and Needleman, P. (1984) Biochem. Biophys. Res. Commun. 121, 802-807.
4. Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazasto, H., Fukuda, A., Minamino, N. and Matsuo, H. (1984) Nature 312, 152-155.

5. Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Eubanks, S.K., Galluppi, G.R. and Needleman, P. (1984) Science 223, 57-63.
6. Seidah, N.G., Lazure, C., Chretien, M., Thibault, G., Garcia, R., Cantin, M., Genest, J., Nutt, R.F., Brady, S.F., Lyle, T.A., Palaveda, W.J., Colton, C.D., Ciccarone, T.M. and Veber, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 2640-2644.
7. Kangawa, K., Fukuda, A. and Matsuo, H. (1984) Nature 313, 397-400.
8. Atlas, S.A., Kleinert, H.D., Camargo, M.J., Januszewicz, A., Sealey, J.E., Laragh, J.H., Schilling, J.W., Lewicki, J.A., Johnson, L.K. and Maack, T. (1984) Nature 309, 717-719.
9. Flynn, T.G., deBold, M.L. and deBold, A.J. (1983) Biochem. Biophys. Res. Commun. 117, 859-865.
10. Misono, K.S., Fukumi, H., Grammer, R.T. and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 119, 524-529.
11. Napier, M.A., Dewey, R.S., Albers-Schönberg, G., Bennett, C.D., Rodkey, J.A., Marsh, E.A., Whinnery, M., Seymour, A.A. and Blaine, E.H. (1984) Biochem. Biophys. Res. Commun. 120, 981-988.
12. Garcia, R., Thibault, G., Seidah, N.G., Lazure, C., Cantin, M., Genest, J. and Chretien, M. (1985) Biochem. Biophys. Res. Commun. 126, 178-184.
13. Ling, N. (1977) Biochem. Biophys. Res. Commun. 74, 248-255.
14. Vuolteenaho, O., Leppäluoto, J., Vakkuri, O., Karppinen, J., Höyhtyä, M. and Ling, N. (1981) Acta. Physiol. Scand. 112, 313-321.
15. Hunter, W.M. and Greenwood, F.C. (1962) Nature 197, 495-496.
16. Bennett, H.P.J., Browne, C.A. and Solomon, S. (1981) Biochemistry 20, 4530-4538.
17. Rossier, J., Vargo, T.M., Minick, S., Ling, N., Bloom, F.E. and Guillemin, R. (1977) Proc. Natl. Acad. Sci. USA 74, 5162-5165.
18. Vuolteenaho, O. (1983) Acta Physiol. Scand. Suppl. 531, 1-84.
19. Vuolteenaho, O., Arjamaa, O., Järvinen, M. and Rinne, A. (1985) Acta Histochem. (in press).
20. Nakao, K., Sugawara, A., Morii, N., Sakamoto, M., Suda, M., Soneda, J., Ban, T., Kihara, M., Yamori, Y., Shimokura, M., Kiso, Y. and Imura, H. (1984) Biochem. Biophys. Res. Commun. 124, 815-821.