Proatrial Natriuretic Factor Is Phosphorylated by Rat Cardiocytes in Culture*

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Proatrial natriuretic factor (proANF) is phosphorylated in primary cultures of neonatal rat cardiocytes. Rittenhouse et al. (Rittenhouse, J., Moberly, L., O'Donnell, M. E., Owen, N. E., and Marcus, F. (1986) J. Biol. Chem. 261, 7607–7610) observed that cyclic AMP-dependent protein kinase phosphorylated synthetic peptides related to atrial natriuretic factor (ANF) and that phosphorylated ANF peptides were more effective in stimulating Na/K/Cl cotransport in smooth muscle cells than nonphosphorylated forms. In our studies, rat cardiocytes in culture were incubated with [32P]orthophosphoric acid, and ANF-related peptides in cell extracts and culture media were isolated using antisera to ANF. Both atrial and ventricular cardiocytes contained and secreted phosphorylated proANF, a 126-amino acid precursor of ANF. Phosphorylated and nonphosphorylated isoforms of proANF were resolved by isoelectric focusing; approximately 35% of the proANF secreted by cardiocytes was phosphorylated. proANF is phosphorylated on a serine residue localized to a 42-amino acid tryptic fragment (proANF residues 26–67). We conclude that proANF is phosphorylated by rat cardiocytes but not within the portion of the molecule destined to become ANF (proANF residues 99–126). Phosphorylation may have a role in the cellular mechanisms of proANF storage and secretion or in the modulation of potential biological activities of the circulating amino-terminal portion of proANF.

Mammalian cardiac tissues synthesize atrial natriuretic factor (ANF), a peptide hormone with potent vasodepressive, natriuretic, and diuretic properties (1, 2). ANF appears to have an important role in maintaining intravascular volume homeostasis and in regulating blood pressure. The hormone is stored in the secretory granules of atrial myocytes as a 126-amino acid precursor, proANF (3, 4). The predominant form of ANF isolated from the circulation is 28 amino acids in length and is derived from the carboxyl terminus of proANF (5). We and others (6, 7) used primary cultures of neonatal rat cardiocytes to investigate the biosynthesis of ANF. Both neonatal atrial and ventricular cardiocytes synthesize and secrete proANF (8).

Recently, Rittenhouse et al. (9) reported that cyclic AMP-dependent protein kinase (protein kinase A) phosphorylated synthetic peptides related to ANF in vitro. In addition, they demonstrated that phosphorylated ANF peptides were more potent than nonphosphorylated forms in stimulating Na/K/Cl cotransport in cultured vascular smooth muscle cells.

In this study, we investigated phosphorylation of proANF using primary cultures of neonatal rat cardiocytes. We demonstrated that proANF is phosphorylated in both atrial and ventricular cardiocytes. The site of phosphorylation in proANF secreted by neonatal rat cardiocytes appears to differ from that in synthetic proANF phosphorylated by protein kinase A.

**MATERIALS AND METHODS**

Phosphorylation of Rat proANF in Neonatal Cardiocytes—Primary cultures of atrial and ventricular cardiocytes were prepared from 1-day-old rats as described previously (8). 5 × 10⁵ cells were plated per 18-mm dish. After 3–5 days in culture, the cells were washed and incubated in phosphate-free balanced salt solution (130 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 20 mM Hepes-Tris buffer, pH 7.4) for 1 h at 37 °C. The culture medium was replaced, and [32P]orthophosphoric acid (0.5 mCi/ml, 1000 mCi/mmol, Du Pont-New England Nuclear) was added. After 3 h the cardiocytes were washed twice and incubated for 2 h in Dulbecco's modified Eagle's medium (without glucose), supplemented with 0.6 mg/ml thymidine and 10% heat-inactivated fetal calf serum. Culture medium was harvested and treated with 1 mM sodium vanadate. Culture medium was similarly harvested from cardiocytes incubated with 200 µCi/ml [38S]methionine (800 Ci/mmol, Du Pont-New England Nuclear).

Synthesis of proANF[2–126]—An analogue of rat proANF, proANF[2–126], was synthesized in Escherichia coli strain W3110 as a fusion protein with β-galactosidase. Expression plasmid construction, fusion protein expression and cleavage, and proANF[2–126] purification will be described in detail elsewhere.1 In brief, a DNA fragment derived from the rat ANF cDNA (10) was inserted into plasmid pUR2 (11) at the 3' end of the bacterial gene encoding β-galactosidase in the correct reading frame. Synthetic DNA linkers were used (i) to introduce a stop codon after tyrosine at position 126 of proANF (see Fig. 2); (ii) to alter the codon for asparagine at proANF position 1 to code for aspartic acid, creating an acid-labile peptide bond between aspartic acid and proline; and (iii) to change the internal Asp-Pro dipeptide at positions 78 and 79 to Asp-Ser. The expressed fusion protein was cleaved between aspartic acid and serine

1 G. Preibisch and G. Seipke, manuscript in preparation.
by treatment with 70% formic acid, as described by Landon (12). After derivatization by oxidative sulfitolysis (13), the fragment mixture was separated by ion exchange chromatography and gel filtration. Folding of proANF[2-126]-S-sulfonate was initiated by addition of β-mercaptoethanol. The final purification by ion exchange chromatography yielded a product which exhibited a single band by SDS-polycrylamide gel electrophoresis. After derivatization by oxidative sulfitolysis (13), the fragment mixture was similarly treated with rat serum (600 μl of culture medium containing 0.25 μg of proANF[2-126]; 300 μl of rat serum).

**Results**

**Protein Kinase A—**

ProANF[2-126] (1 μg) was phosphorylated in 0.1 M potassium phosphate (pH 7.1), 10 mM MgCl₂, 20 mM ATP containing [γ-³²P]ATP (50 μCi/ml, Du Pont-New England Nuclear), and 65 μg/ml catalytic subunit of protein kinase A, isolated from bovine heart as described by Beavo et al. (14). After a 30-min incubation at 37 °C, the reaction was terminated by addition of EDTA to a final concentration of 25 mM.

**Generation of proANF Cleavage Products—**

Cardioocyte culture medium containing radiolabeled proANF (approximately 1 ng/ml) was incubated (21) with rat serum for 30 min at 37 °C, as previously described (15). proANF[2-126], phosphorylated by protein kinase A, was similarly treated with rat serum (600 μl of culture medium containing 0.25 μg of proANF[2-126]; 300 μl of rat serum).

**Immunoprecipitation and Gel Electrophoresis—**

As previously described (7, 15), radiolabeled proANF-derived peptides were incubated with antisera raised against proANF residues 57-68 and residues 118-126 (proANF[57-68] and proANF[118-126], respectively). Peptide-antibody complexes were isolated by adsorption to Staphylococcus aureus cells (Pansorbin, Behring Diagnostics). Following washing, the complexes were eluted in 3% SDS and 5% β-mercaptoethanol. The eluate was fractionated by electrophoresis on a 17% polyacrylamide gel containing SDS; gels were examined by autoradiography. The molecular weights of the radiolabeled peptides were estimated by comparison with protein standards (Bethesda Research Laboratories). (For preparative gel electrophoresis, cardiocyte culture medium containing [³²P]-labeled proANF was incubated with S. aureus cells for 2 h at 4 °C. proANF was immunoprecipitated from the adsorbed culture medium as described above.)

**Phosphorylation and Trichloroacetic Acid Precipitation—**

For isoelectric focusing, immune complexes were eluted in 0.6 g/100 ml 8% Triton X-100, and 7.5% ammonium oxalate (equal volumes of Bio-Lyte 5/7 and Bio-Lyte 3/10, Bio-Rad). The eluate was fractionated on an isoelectric focusing gel slab containing equal volumes of Bio-Lyte 5/7 and Bio-Lyte 3/10 at 750 V h. Gels were fixed and autoradiographed. The isoelectric points of the radiolabeled peptides were estimated by comparison to the pH of amphotolysates eluted in water from 1-cm² gel slices (16). To compare quantities of radioabeled peptides, autoradiographs were scanned on an LKB 2222-010 Ultrascan Laser Densitometer equipped with the LKB 2400 Gelscan software package.

**Phosphoamino Acid Analysis—**

Radiolabeled polyacrylamide gel electrophoresis, [³²P]-labeled proANF from cardioocyte culture medium was recovered by electroelution as described by Hunkapiller et al. (17). Bovine serum albumin (50 μg) was added, and the proteins were precipitated with 10 volumes of ethanol. The proteins were resuspended in 0.2 ml of 50 mM ammonium bicarbonate, pH 8.3, and heated at 100 °C for 4 h. The resulting phosphoamino acids were analyzed by one-dimensional thin layer chromatography as described by Hunter and Setton (18).

**Trypic Tryptic Peptide Mapping—**

Electroeluted [³²P]-labeled proANF from cardioocyte culture medium was reduced with dithiothreitol and carboxymethylated as described by Kranget al. (19). Electroeluted [³⁵S]methionine-labeled proANF was similarly treated and was mixed with [³²P]-labeled proANF. Bovine serum albumin (50 μg) was added, and the proteins were precipitated with ethanol. The proteins were resuspended in 50 mM ammonium bicarbonate, pH 8.3. Tosylphenylalanyl chloromethyl ketone trypsin (Worthington, 2.5 pg total) was added in three aliquots at 0, 3, and 12 h, and the reaction mixture was incubated at 37 °C for 16 h. Tryptic peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) at 40 °C on a Vydac 218 TP446 ODS column equilibrated with 0.1% trifluoroacetic acid in water. After sample application, the column was washed for 5 min with the starting solvent and eluted with a 95-5 min gradient of 0-50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.4 ml/min. Fractions of 0.7 ml were collected, and those containing [³²P] identified by measuring Cerenkov radiation. Fractions containing [³⁵S]-labeled peptides were identified by liquid scintillation spectrometry. The ratio of [³⁵S] to [³²P] cpm was such that the contribution of [³²P] to the [³⁵S] cpm was negligible.

proANF[2-126] phosphorylated by protein kinase A was isolated by SDS-polycrylamide gel electrophoresis. Following carboxymethylation and trypsin digestion, the resulting radiolabeled peptides were analyzed by reverse-phase HPLC, as above.

**Localization of the Site of Phosphorylation in proANF Isolated from Cardiacocytes—**

Characterization of the amino acid sequence surrounding the site of phosphorylation in proANF may facilitate identification of the protein kinase which phosphorylates it in vivo. In order to identify the amino acid of proANF which is phosphorylated in rat cardioocytes, [³²P]-labeled proANF was subjected to acid hydrolysis, and the resulting phosphoamino acids were examined by thin layer chromatography. The predominant phosphoamino acid was...
Phosphorylation of proANF

Fig. 1. Immunoprecipitation of 32P-labeled proANF. Panel A, proANF phosphorylated in cardiocytes; atrial cardiocytes were incubated with [32P]orthophosphoric acid for 3 h. Cells were washed and then incubated in nonradioactive Dulbecco’s modified Eagle’s medium with fetal calf serum. After 2 h, the culture medium was harvested. A sample of culture medium was mixed with rat serum, in a ratio of 2:1, and incubated at 37 °C for 30 min. Treated (incubation with serum) and untreated (control) samples of culture medium were reacted with normal rabbit serum (NRS) and with antisera against synthetic peptides proANF[57–68] (lanes marked 1) and proANF[118–126] (lanes marked 2). Panel B, proANF phosphorylated by protein kinase A; proANF[2–126] was phosphorylated in the presence of [γ-32P]ATP with protein kinase A. One sample of the reaction mixture was mixed with rat serum as above. Treated (incubation with serum) and untreated (control) samples were reacted with normal rabbit serum and with antisera against the proANF peptides, as in panel A.

Fig. 2. Amino acid sequence of rat proANF. The structure of ANF, isolated from rat plasma, is shown in capital letters (5). Sites of proANF cleavage (*) upon mixture with serum are indicated (15). Tryptic peptides containing methionine residues (bold) are underlined (T1 and T3). T12, the tryptic peptide containing Ser103, is also underlined. Potential phosphorysineines (*) are indicated within T3, the trypic fragment phosphorylated by atrial cardiocytes. The tryptic peptides are numbered according to Kangawa et al. (4).

32P-Labeled proANF and [35S]methionine-labeled proANF were isolated from atrial cardiocyte culture media and subjected to trypsin digestion. The resulting tryptic peptides were analyzed by reverse-phase HPLC. As expected from the amino acid sequence of proANF (Fig. 2), trypsin digestion of [35S]methionine-labeled proANF generated two radiolabeled peptides (Fig. 3, panel B). Kangawa et al. (4) subjected proANF isolated from rat atria to trypsin digestion and fractionated the resulting peptides by reverse-phase HPLC. They showed that the more polar methionine-containing tryptic peptide (designated T1) consisted of proANF residues 1–16 and the more hydrophobic peptide (designated T3) consisted of proANF residues 26–67. All other tryptic peptides of proANF eluted prior to T1 and T3 on reverse-phase HPLC. Tryptic digestion of 32P-labeled proANF resulted in a single predominant 32P-labeled peptide which eluted immediately before 35S-labeled T3 (Fig. 3, panel C), suggesting that it was a phosphorylated form of T3.

To confirm that the major 32P-labeled tryptic fragment was T3 (proANF[26–67]) culture medium from atrial cardiocytes incubated in the presence of [32P]orthophosphoric acid was digested with trypsin, immunoprecipitated with antiserum to proANF[57–68], and fractionated by SDS-polyacrylamide gel electrophoresis. A 32P-labeled peptide, approximately 5 kDa in size, was immunoprecipitated (data not shown). A peptide with similar electrophoretic mobility was isolated from trypsin-treated culture medium containing [35S]methionine-labeled proANF. We conclude that 32P-labeled proANF was predominantly phosphorylated at 1 or more of 5 serine residues within T3.

Other fractions containing 32P radioactivity were evident upon HPLC fractionation of the 32P-labeled proANF trypic digest. Radioactivity eluting prior to fraction 10 represented free orthophosphoric acid contained in the starting material. The other 32P-labeled substances were not identified. They may represent other tryptic fragments of proANF, tryptic fragments of contaminating phosphopeptides, or, more likely, nonspecifically degraded products of phosphorylated T3.

Trypsin Digestion of proANF[2–126] Phosphorylated by Protein Kinase A—To further compare proANF phosphorylated in rat cardiocytes with that phosphorylated by protein kinase A, 32P-labeled proANF[2–126] was isolated by SDSPolyacrylamide gel electrophoresis and digested with trypsin. Reverse-phase HPLC fractionation of the resulting radiolabeled peptides showed that the 32P radioactivity was associated with tryptic peptides more polar than 35S-labeled T1 and T3 (Fig. 3, panel D). Evidence that protein kinase A phosphorylated synthetic ANF peptides at Ser103 (9) and phosphorylated proANF[2–126] at a residue within its carboxyl-terminal 28 amino acids suggested that protein kinase A phosphorylated proANF[2–126] at Ser103. The 32P-labeled tryptic peptides derived from proANF[2–126] probably represent, therefore, proANF residues 103–109 (Fig. 2, T12) and the product of incomplete digestion at Arg102-Ser103, proANF residues 102–109 (arg-T12). Phosphorylation of proANF in rat cardiocytes at a residue within T12 was not completely ex-
**FIG. 3. Localization of phosphoamino acid in $^{32}$P-labeled proANF.** Panel A, phosphoamino acid analysis. $^{32}$P-Labeled proANF, isolated from atrial cardiocyte culture medium, was hydrolyzed by heating in 6 N HCl. The resulting phosphoamino acids were analyzed by thin layer chromatography. Marker standards for phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) are indicated. Panels B, C, and D, reverse-phase HPLC analysis of tryptic peptides derived from $^{[35]S}$methionine-labeled proANF (panel B), $^{32}$P-labeled proANF, secreted by cardiocytes (panel C) and $^{32}$P-labeled proANF[2-126], phosphorylated by protein kinase A (panel D). Radiolabeled proANF was digested with trypsin, and the resulting tryptic peptides were fractionated by reverse-phase HPLC. Two $^{[35]S}$methionine-labeled tryptic peptides are predicted from the amino acid sequence of proANF (see Fig. 2, T1 and T3). The order of elution of these peptides has been established by Kangawa et al. (4). The predominant $^{32}$P-labeled tryptic peptide derived from proANF secreted by rat cardiocytes eluted just prior to the $^{35}$S-labeled T3. The radiolabeled tryptic peptides derived from proANF[2-126], phosphorylated by protein kinase A, are clearly distinct from $^{35}$S-labeled T1 and T3 and may well represent T12 and the product of incomplete digestion at Arg$^{102}$-Ser$^{103}$, arg-T12 (proANF[102-109]).
cluded; small amounts of $^{32}$P radioactivity coeluting with T12 were found upon HPLC fractionation of trypsin-digested $^{32}$P-labeled proANF.

Isoelectric Focusing of proANF—To estimate the percentage of proANF secreted by cardiocytes which was phosphorylated, [35S]methionine-labeled proANF and $^{32}$P-labeled proANF from atrial cardiocyte culture media were characterized by isoelectric focusing. Two isoforms of proANF were identified with isoelectric points estimated to be 5.6 and 5.4 (Fig. 4, $^{35}$S, lane A). Both isoforms were precipitated by the two noncross-reactive antisera to proANF and were cleaved by incubation with serum suggesting that they were both proANF. Only the isoform with the more acidic isoelectric point was radiolabeled in cardiocytes incubated with $[^{32}P]$orthophosphoric acid (Fig. 4, $^{32}P$, lane A). The isoform with the more basic isoelectric point appeared to be the nonphosphorylated form of proANF. The phosphorylated isoform of proANF represented approximately 35% of the total proANF secreted by atrial cardiocytes in culture.

proANF Is Phosphorylated in Neonatal Ventricular Cardiocytes—In order to identify any possible structural differences in proANF secreted by neonatal atrial and ventricular cardiocytes, phosphorylation of proANF by ventricular cardiocytes was examined. Ventricular cardiocytes were incubated with $[^{32}P]$orthophosphoric acid, and the radiolabeled peptides from cell extracts and culture media were immunoprecipitated with antisera to proANF. Phosphorylated proANF was contained in and secreted by ventricular cells (data not shown). Identification of a 5-kDa $^{32}$P-labeled peptide in trypsin-treated ventricular cardiocyte culture medium further suggested that ventricular proANF was also phosphorylated within the trypic fragment T3. Isoelectric focusing of proANF secreted by ventricular cardiocytes revealed a ratio of phosphorylated to nonphosphorylated isoforms similar to that found for proANF secreted by atrial cardiocytes (Fig. 4, $^{32}P$ and $^{35}$S, lanes V).

**DISCUSSION**

Neonatal rat atrial and ventricular cardiocytes in culture contain and secrete phosphorylated proANF. Acid hydrolysis of $^{32}$P-labeled proANF identified a serine residue as the site of phosphorylation. The location of the phosphoryserine in proANF was characterized using two complementary approaches. Incubation of $^{32}$P-labeled proANF with rat serum generated a radiolabeled 14-kDa cleavage product suggesting that proANF was phosphorylated within its amino-terminal 98 amino acids. Tryptic mapping of $^{32}$P-labeled proANF further localized the phosphoryserine to a 42-amino acid fragment, proANF residues 26–67. If phosphorylation were critical for a physiological function of proANF, conservation of phosphorylated residues and the surrounding amino acid sequences might be expected as the species diverged during evolution. Only two of the five serine residues in the trypic fragment are conserved [Ser29 and Ser50] in all six species (bovine (20), dog (21), human (22), mouse (22), rabbit (21), and rat (10)) for which the amino acid sequences of proANF are known.

We suggest that phosphorylation of proANF is likely to occur at serine residue 39 or 50.

Protein kinase A phosphorylated synthetic proANF[2–126] within its carboxyl-terminal 28 amino acids, most probably at Ser104. Protein kinase A is present in rat myocardial tissue (23), and it might, therefore, be expected that proANF would be phosphorylated at Ser104 in cultured cardiocytes. Failure to find appreciable amounts of proANF phosphorylated at Ser104 by cardiocytes may be attributed to localization of proANF and protein kinase A to different cellular compartments. Alternatively, it is possible that protein kinase A phosphorylates proANF in cardiac cells only under certain conditions. In preliminary experiments, we attempted to stimulate protein kinase A activity in atrial cardiocytes by increasing the intracellular cyclic AMP concentration (24–26). In the presence of forskolin or 8-bromo-cyclic AMP and 3-isobutyl-1-methylxanthine, atrial cardiocytes did not incorporate $^{32}$P into the 3-kDa proANF cleavage product (containing Ser104) (data not shown). We conclude that proANF secreted by rat cardiocytes is phosphorylated at Ser104 at low levels if at all. We suggest, therefore, that the portion of proANF destined to become the circulating form of ANF, proANF[99–126], is unlikely to be phosphorylated to any significant extent.

Phosphorylation is critical to the regulation of many cellular processes including differentiation and proliferation. Several peptide hormones are phosphorylated by isolated protein kinases (cited in Ref. 9). Phosphorylation of peptide hormones in intact cells has been demonstrated for prolactin (27), for peptides derived from pro-opiomelanocortin (16), and for the alpha subunit of human chorionic gonadotropin (28). A physiological role for the phosphorylation of peptide hormones remains to be defined. One hypothesis is that phosphorylation of peptide hormones might be involved in the cellular mechanisms of peptide compartmentalization and secretion. Phosphorylation of oligosaccharide chains appears to be important for the correct sorting of lysosomal enzymes into lysosomes (29). Endocrine cells, in general, sequester peptide hormones into secretory granules, where the peptides are stored at high concentration available for bolus release in response to stimuli (regulated secretory pathway (30)). Atrial cardiocytes are able to store and secrete proANF via a regulated pathway (8). Neonatal ventricular cells, on the other hand, have only rare secretory granules and secrete proANF rapidly after synthesis (constitutive secretory pathway (30)). The secretion of phosphorylated and nonphosphorylated proANF isoforms by ventricular cardiocytes suggests that phosphorylation of proANF alone is not sufficient for regulated secretion. It remains to be determined if phosphorylation of proANF is important for sorting or storage within the secretory granules of atrial cardiocytes.

Recently, Michener et al. (31) have reported that a 14-kDa peptide derived from the amino terminus of proANF can be isolated from rat plasma. Although the biological function of this proANF metabolite is unknown, our results suggest that some percentage of the circulating 14-kDa peptides is phos-
Phosphorylated. It is, therefore, possible that phosphorylation may regulate the biological activity of this circulating 14-kDa proANF metabolite.

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