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Proteolytic Processing of Atriopeptin Prohormone

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SUMMARY

The metabolism of atriopeptin prohormone ANF1-126 was examined with the aid of two separate radioimmunoassays, one detecting the C-terminal atriopeptins and the other detecting a fragment of the prohormone N-terminus. Intact prohormone standards are recognized in both assays, whereas the C-terminal atriopeptins are only detected by the atriopeptin assay. Both atriopeptin and N-terminal fragment immunoreactivities were detected in rat plasma and were simultaneously elevated following intravenous administration of desamino-arginine-vasopressin. Atriopeptin immunoreactivity returned to basal levels within 60 min after desamino-arginine vasopressin administration, whereas the N-terminal fragment immunoreactivity remained elevated for more than 2 hr. Analysis of both acid-boiled and sodium dodecyl sulfate-boiled rat atrial extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting revealed the presence of a single high molecular weight species which reacted to both antisera and which comigrated with atriopeptin prohormone standards. Western blots of plasma from desamino-arginine vasopressin-stimulated rats yielded both the low molecular weight C-terminal atriopeptin and a high molecular weight N-terminal fragment-reactive peak which was smaller than the prohormone standards and which did not possess atriopeptin immunoreactivity. A recombinant 128-amino acid atriopeptin prohormone construct, ANF1-126-Arg-Arg, was used as a model substrate for prohormone metabolism. ANF1-126-Arg-Arg was specifically cleaved followed incubation with thrombin to yield the 98-amino acid N-terminal fragment and the C-terminal atriopeptin, AP28-Arg-Arg. Processing of ANF1-126-Arg-Arg by reperfusion through an isolated heart or by incubation in serum yielded identical metabolites to those generated by incubation with thrombin. No significant metabolism was observed following incubation of the prohormone with rat plasma. We conclude that the rat heart contains the necessary enzyme to cleave both endogenous and exogenous prohormone to atriopeptin and that processing by blood enzymes is not required.

The observation by DeBold *et al.* (1) that rat atria contain a peptide which is a potent natriuretic and diuretic led to the discovery of a novel hormonal system which participates in salt and water homeostasis. In addition to being diuretic and natriuretic, this hormone (AP) possesses spasmolytic activity toward smooth muscle (2) and inhibitory effects on aldosterone (3–6), vasopressin (7), and renin secretion (8, 9). AP is stored within rat atrial myocytes as a 126-amino acid prohormone (10), termed ANF1-126, and is released into the circulation from the atria in response to atrial stretch (11, 12). During, or immediately after secretion, ANF1-126 undergoes proteolysis to yield the circulating form of the hormone, the 28-amino acid C-terminal peptide, AP28) (13, 14). Little is known about the location and identity of the enzymes that are responsible for this proteolysis. Isolated perfused rabbit hearts have been shown to secrete primarily a low molecular weight AP (12, 15). It is not clear whether the prohormone is secreted intact (16, 17) or whether it is processed by the time it leaves the myocyte (18). Intact AP126 has not been detected in circulating blood or in the coronary venous effluent of isolated perfused hearts. Both platelets (19) and serum (16) have been proposed as potential sites for prohormone processing. Kallikrein has also been demonstrated to convert crude high molecular weight APs to the more biologically active low molecular weight APs (20).

In order to study the metabolism of ANF1-126, we have developed two separate RIAs. The first, which has been described previously (14), recognizes both high molecular weight AP the low molecular weight AP24 and AP28. The second assay uses antisera directed against a synthetic 20-amino acid peptide corresponding to a portion of the NTF (termed NTF48-67) of ANF1-126. We have also used a recombinant AP prohormone substrate to examine potential sites for prohormone to hormone conversion.

Materials and Methods

Peptide synthesis. Peptides were synthesized by solid phase synthesis on an Applied Biosystems Peptide Synthesizer model 430-A and purified by HPLC (21). Coupling of the peptides to bovine thyroglobulin

ABBREVIATIONS: AP, atriopeptin; RIA, radioimmunoassay; HPLC, high pressure liquid chromatography; EDTA, ethylenediaminetetraacetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; APT, aminophenylthioether; NTFir, N-terminal immunoreactivity (-reactive); dAVP, desamino-arginine-vasopressin; APir, atriopeptin immunoreactivity (-reactive).

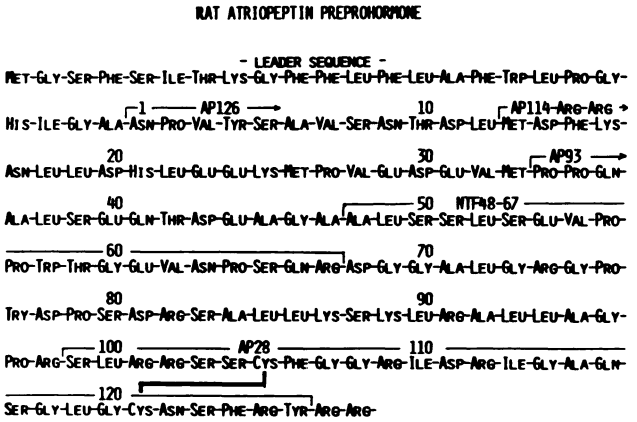

Fig. 1. Amino acid sequence for rat atriopeptin pre-prohormone. The first 24 amino acids on the N-terminus represent the hydrophobic leader sequence. AP126 (ANF1-126), the atrial storage form, runs from Asn[1] to Tyr[126]. Fragments of AP126 are designated by AP followed by their amino acid numbers. Atriopeptins having Tyr[126] as C-terminal are designated by AP followed by the total number of amino acids.

was accomplished using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (22) as a cross-linking reagent.

Prohormone preparation. ANF1-126-Arg-Arg, representing amino acid residues 25–152 of the rat pre-prohormone coding sequence (10), was produced in *Escherichia coli* using recombinant DNA techniques. The amino acid sequence for ANF1-126-Arg-Arg was confirmed by gas phase sequence analysis and the polypeptide was determined to be biologically active both *in vivo* and *in vitro*.[1] ANF1-126-Arg-Arg was chosen as an exogenous substrate for the prohormone to hormone activating enzyme(s) because it contained the complete amino acid sequence for the putative cleavage site (Arg^{98}-Ser^{99}). ANF1-126-Arg-Arg also contains the two C-terminal arginine residues coded for by the genomic cDNA which are absent from the atrial storage form (ANF1-126).

Atriopeptin radioimmunoassay. Male New Zealand rabbits were immunized against a cyanogen bromide-generated AP prohormone fragment containing amino acids 34–126 (AP93, Fig. 1) (23). Rabbit 11 yielded an antiserum having the greatest sensitivity and a titer of 1:5,000. Radiolabeled AP24 was prepared by iodination using a modification of the chloramine T method. Monoiodo-[^{125}I]AP24 was separated from the unreacted AP24 and the diiodo peptide by reverse phase HPLC using a μBondapak C-18 column and a linear gradient of acetonitrile/water with 0.05% trifluoroacetic acid. [^{125}I]AP24 fractions were stored in RIA buffer at −20°. RIA samples were incubated with buffer (100 mM sodium phosphate, pH 7.4, 0.25% bovine serum albumin, 10 mM sodium azide, 3% polyethylene glycol), 50 μl of antiserum 11 (1:5,000 final dilution), 50 μl of [^{125}I]AP24 (10,000 cpm/tube), and 20 μl of goat anti-rabbit IgG (1:50 final dilution) for a final assay volume of 300 μl. Assays were incubated overnight at 4°, diluted with 2 ml of 0.25% bovine serum albumin and centrifuged at 1000 × *g* for 30 min; then, the resulting pellet was counted in a Micromedics 10-channel gamma counter. The antibody concentration used resulted in 40% total specific binding.

N-Terminal fragment radioimmunoassay. Synthetic NTF48-67 (Fig. 1) was covalently linked to bovine thyroglobulin and used to immunize guinea pigs. A guinea pig responded with a high affinity antiserum having a titer of 1:25,000. Synthetic N-Tyr-NTF48-67 was iodinated as described above and purified by reverse phase HPLC. Assays consisted of 160 μl of sample with buffer [50 mM sodium phosphate, pH 6.8, 10 mM EDTA, 0.3% bovine gamma globulin, 10 mM sodium azide, 0.005% Triton X-100, 20 μl of antisera (1:2,500 dilution)] and 20 μl of [^{125}I]NTF48-67 (10,000 cpm). Assays were incubated at 4° overnight, after which the antibody complexes were precipitated by addition of 16% polyethylene glycol. Supernatants were decanted and the pellets were counted in a gamma counter. The antibody concentration used resulted in 40% total specific binding.

Blood pressure and plasma samples. Male Sprague-Dawley rats (300–400 g) were anesthetized with 5 ml/kg 7% chloral hydrate. PE-50 polyethylene catheters were inserted into the carotid artery for measurement of blood pressure, the jugular vein for infusion of peptides, and the femoral artery for collection of blood samples. Animals were allowed to stabilize for 20 min before starting the experiments. Blood pressure was measured by means of a COBE transducer connected to a Beckman R611 physiograph. Drugs were dissolved in saline for injection. Blood samples were collected into 1 part sodium citrate (110 mM) to 10 parts blood and immediately centrifuged; the plasma was collected, frozen on dry ice, and stored at −70° until assayed by RIA.

Blood used for *in vitro* prohormone conversion experiments was obtained from the abdominal aorta. Plasma was generated by collecting the blood into EDTA for a final concentration of 1.5 mg/ml. Blood for serum was collected without EDTA, allowed to clot, and centrifuged, and the serum was separated. EDTA was then added to the serum to match the concentration in the plasma.

Isolated perfused rat hearts. Chloral hydrate-anesthetized male Sprague-Dawley rats were given 100 units of heparin intravenously. Thirty min later, rats were sacrificed, and their hearts were dissected out and immediately placed in ice-cold Krebs buffer. Polyethylene shunts (PE-50) were inserted into both ventricles and the aorta was cannulated for retrograde perfusion. Hearts were perfused at 10 ml/min with oxygenated Krebs buffer (95% O_2/5% CO_2), and 37°, and were allowed to equilibrate for 60 min. Preparation for the reperfused hearts was the same except that the perfusate was collected in a small reoxygenation chamber from which it was pumped back through the heart. The entire reperfusion system was siliconized before use. Equilibration consisted of four changes of the reperfusate for 60 min (total volume = 30 ml).

Atrial extracts. Rat hearts were quickly removed and rinsed with ice-cold saline. Atria were removed, quick frozen on dry ice, and then powdered using a liquid nitrogen-cooled stainless steel piston. The powdered atria were then extracted by boiling in acetic acid or by boiling in sample buffer for SDS-PAGE. Acid extracts were performed by adding 1 ml of 1 N acetic acid heating at 100% for 10 min, and homogenizing with a Polytron homogenizer. Homogenates were centrifuged for 20 min at 28,000 × *g* Supernatants were stored at −20° until assayed.

Immunoaffinity extraction. IgG from anti-NTF48-67 antiserum from anti-AP24 antiserum were separately purified using a Bio-Rad Affi-Gel Protein A affinity column, concentrated, and then were each covalently coupled to Bio-Rad Affi-Gel 10 activated affinity supports. These affinity columns were then equilibrated with 20 volumes of buffer containing 25 mM Tris (pH 7.9) and 150 mM NaCl before loading the samples. Nonspecific proteins were removed with 20 volumes of 25 mM Tris (pH 7.4), 500 mM NaCl, 0.2% Triton X-100, followed by 10 volumes of 150 mM NaCl, 0.1% Triton X-100, and 5 volumes of dH_2O. Samples were then eluted with 1 N acetic acid and lyophilized.

Western blotting. SDS-PAGE separation of samples was performed according to the method of Laemmli (24). Two identical gels were prepared for each sample for blotting by two different antisera. Proteins from these gels were electrotransferred and covalently bound onto separate diazotized APT cellulose papers (Schleicher & Schuell) for immunoblotting with either atriopeptin antisera or N-terminal fragment antisera. For immunoblotting, transfer papers were first incubated for 1–2 hr at 37° in 100 mM Tris (pH 9.0) and 0.25% gelatin to inactivate the remaining diazo groups, then rinsed with water. Transfer papers were then incubated for 2–12 hr at room temperature in 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 0.25% gelatin, 0.05% Nonidet P-40, and either AP (a separate antiserum directed against AP24 was used for Western blotting) or NTF antisera (1:3000). Nonspecific antibody binding was washed off and the specifically bound antibody was visualized by incubating the papers with ^{125}I-protein A

[1] M. L. Michener *et al.*, unpublished results.

(Amersham) (25 μCi/lane). Papers were washed to remove nonspecific ^{125}I-protein A binding, dried, and exposed to Kodak XAR-5 film at −70°.

Results

Immunoassays. An immunoassay for rat AP was developed which detects both the stored AP126 prohormone and the circulating AP28. AP24 competes with [^{125}I]AP24 for binding to this antisera with a half-maximal concentration of 30 fmol/tube (Fig. 2A). AP28 reacts equally well compared to AP24 (data not shown). Two prohormone fragments, a cyanogen bromide fragment, ANF34-126 and a recombinant fragment, ANF13-126-Arg-Arg, both of which possess an intact C-terminus, gave parallel competition curves to AP24, whereas the synthetic 20-amino acid peptide, corresponding to a portion of the NTF of rat AP prohormone, NTF48-67, did not cross-react (less than 0.1%).

NTF48-67 was used to generate antibodies which would recognize the prohormone but not the biologically active C-terminal 28-amino acid peptide (AP28). Unlike AP28, NTF48-67 does not possess natriuretic, diuretic, or spasmolytic activities (data not shown). Thyroglobulin-coupled NTF48-67 generated high affinity antiserum in guinea pigs. N-Tyr-NTF48-67 was ^{125}I labeled for use as a radioligand. Sixty fmol of NTF48-67 produced a half-maximal competition of [^{125}I]Tyr-NTF48-67 from the antiserum at a final dilution of 1:25,000 (Fig. 2B). This antiserum did not cross-react with the C-terminus peptide AP24 but did cross-react with both the 93-amino acid cyanogen bromide fragment AP93 (57%) and the recombinant 116-amino acid polypeptide, AP114-Arg-Arg (54%).

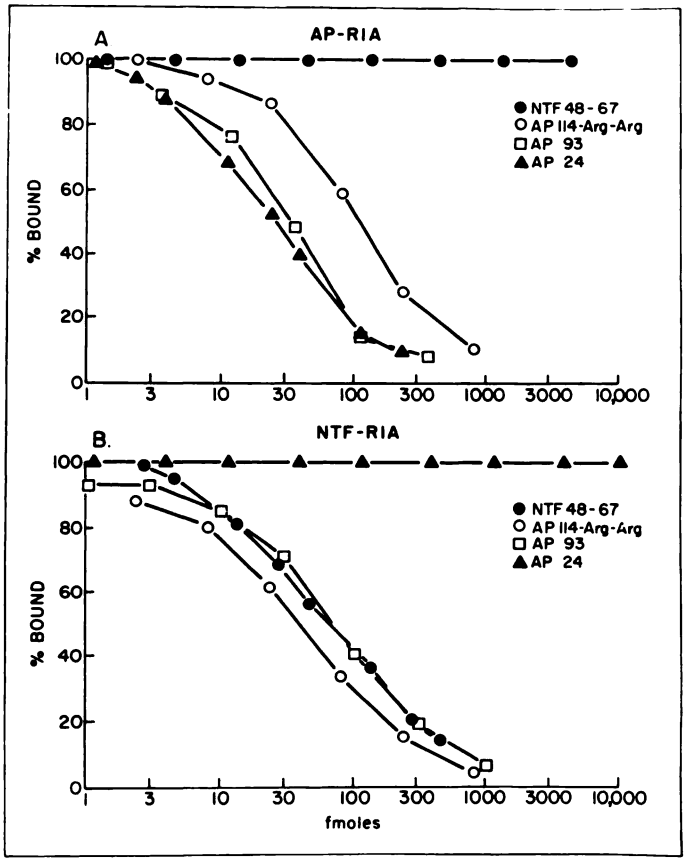

Fig. 2. A. Atriopeptin RIA (*AP-RIA*). Competition curves of various peptides for displacement of [^{125}I]AP24 for binding to rabbit AP antisera are shown. B. NTF-RIA. Competition curves for displacement of [^{125}I]-N-Tyr-NTF48-67 from guinea pig NTF antisera.

Detection of plasma NTFir. Previous work from this laboratory has already demonstrated that the vasopressin analog, dAVP, when injected into rats, causes an increase in plasma APir (14). We reasoned that, since the C-terminal peptide from AP126 was found in the plasma, then the N-terminus would also be present in plasma. To determine whether the remaining N-terminal portion of the prohormone is also released into the circulation, Sprague-Dawley rats were injected with 3 μg of dAVP intravenously and subsequent blood samples were obtained for determination of both plasma APir and NTFir (Fig. 3). Plasma APir was elevated from a basal value of 0.28 ± 0.08 pmol/ml at t = 0 min to a maximum of 2.35 ± 0.16 pmol/ml at t = 5 min. Diastolic blood pressure elevation in response to dAVP followed a time course similar to that of plasma APir, having a maximal elevation from 77 ± 4.4 mm Hg to 107 ± 6.3 mm Hg in 5 min. Both APir and diastolic blood pressure returned to basal values after 60 min. Basal NTFir was found to be much higher (2.14 ± 0.40 pmol/ml) than basal APir. NTFir was also elevated following dAVP stimulation but did not reach the peak value of 6.46 ± 0.42 pmol/ml until 60 min after injection. NTFir was still elevated after 120 min (5.02 ± 1.07 pmol/ml).

Characterization of NTFir in atrial extracts. To characterize the NTFir, both acetic acid and SDS-PAGE extracts of rat atria were prepared and analyzed by Western blotting. These extracts, together with AP standards, were run on two identical SDS-PAGE gels and electrotransferred to separate APT papers. One paper was blotted with an anti-AP24 antiserum (Fig. 4, blot A) and the other with an anti-NTF48-67 antiserum (Fig. 4, blot B). Lanes *1A* and *1B* in Fig. 4 contain both synthetic AP28 (3 kDa) and the recombinant prohormone ANF1-126-Arg-Arg (18 kDa). As expected, the prohormone reacts with both antisera, whereas AP28 is only detected by the anti-AP antiserum. Incubation of ANF1-126-Arg-Arg with thrombin completely cleaves the prohormone to generate the AP-immunoreactive AP28-Arg-Arg (Fig. 4, lane *2A*, which has a migration similar to that of AP28 (3 kDa), and the NTF-

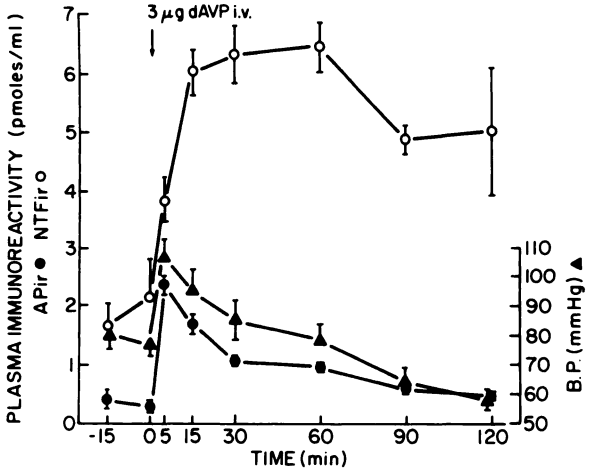

Fig. 3. Time course of rat plasma APir and NTFir following bolus intravenous administration of 3 μg of dAVP. Three μg of dAVP in saline were injected intravenously at 0 min. Plasma was assayed for APir (●) and NTFir (○) together with mean arteriolar blood pressures (*B.P.*, ▲). Values are the mean ± standard error, n = 4.

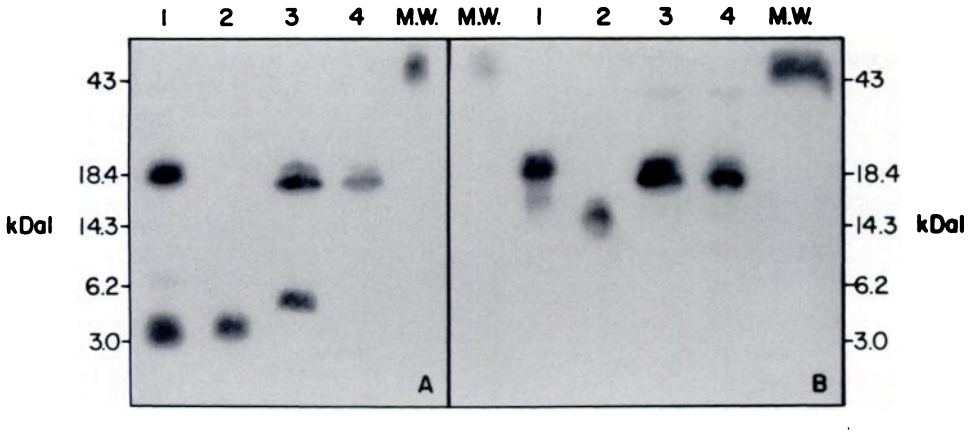

Fig. 4. Western blot of atriopeptin standards and atrial extracts. Samples and standards were run on two identical 15% SDS-PAGE gels and electrotransferred to separate APT papers. One paper was blotted with an AP24 antiserum (blot A) and the other with an NTF48-67 antiserum (blot B). Lanes *1A* and *1B*, mixture of 0.5 μg of AP28 and 0.5 μg of ANF1-126-Arg-Arg; lanes *2A* and *2B*, 0.5 μg of thrombin-cleaved ANF1-126-Arg-Arg; lanes *3A* and *3B*, a 1 N HOAc extract of rat atria; lanes *4A* and *4B*, SDS-boiled atrial extract. Antibodies were detected with ^{125}I-protein A and autoradiography. High molecular weight bands in the molecular weight (*M.W.*) lanes represent ^{125}I-protein A binding to the ovalbumin standard.

immunoreactive 98-amino acid metabolite (Fig. 4, lane *2B*), which migrates as a 14-kDa polypeptide. The site of this cleavage has been confirmed by sequence analysis of the metabolites (data not shown) and was found to be specifically at the Arg^{98}-Ser^{99} bond of ANF1-126.

Both acetic acid (Fig. 4, lanes *3A* and *3B*) and SDS (lanes *4A* and *4B*) extraction of cold powdered rat atria yield a predominant prohormone species which comigrates with ANF1-126-Arg-Arg and reacts with both antisera. Acid extraction also produced a 6-kDa AP-immunoreactive species (Fig. 4, lane *3A*) which was not seen in the SDS extract (Fig. 4, lane *4A*).

Characterization of plasma NTFir. Plasma obtained from rats 3 min after an intravenous bolus of 3 μg of dAVP was also processed for analysis by Western blot (Fig. 5). In contrast to the Western blot of the rat atrial extract, rat plasma displayed only a single APir band (Fig. 5, lane *3A*), which comigrated with AP28-Arg-Arg (Fig. 5, lane *2A*). High molecular weight APir was not detected by the AP blot. High molecular weight NTFir was found predominantly in a single band at 14 kDa (Fig. 5, lane *3B*), which is where the 98-amino acid N-terminal thrombin cleavage product of the prohormone migrated (Fig. 5, lane *2B*). The NTFir band showed no detectable APir and did not comigrate with the prohormone, indicating that the NTFir fragment lacked part or all of the C-terminal hormone sequence. Only a trace of intact prohormone was detected by the NTF antiserum in Fig. 5, lane *3B*.

Metabolism of exogenous prohormone by isolated perfused hearts. To see whether exogenous prohormone could also be processed by the isolated heart, an apparatus was set up so that Krebs buffer could be reperfused through the heart. The prohormone substrate ANF1-126-Arg-Arg was injected (15 μg) into the perfusion line and allowed to recirculate for 20 min. The reperfusate (20 ml) was then collected into a mixture of protease inhibitors and extracted separately on consecutive NTF (Fig. 6, lanes marked *n*) and AP (Fig. 6, lane marked *a*) affinity columns. Affinity extracts were then analyzed on Western blots, one with AP antisera (Fig. 6, blot A) and the other with NTF antisera (Fig. 6, blot B). AP affinity extracts (Fig. 6A) and NTF affinity extracts (Fig. 6B) for each sample were run in parallel lanes.

Addition of ANF1-126-Arg-Arg to the reperfused heart for 20 min resulted in greater than 50% conversion of prohormone to a 3-kDa APir band (Fig. 6, lane 1*a*, blot A) and a 14-kDa NTFir band (Fig. 6, lane 1*n*, blot B). Control reperfusate (fresh Krebs buffer reperfused for 20 min through the isolated heart

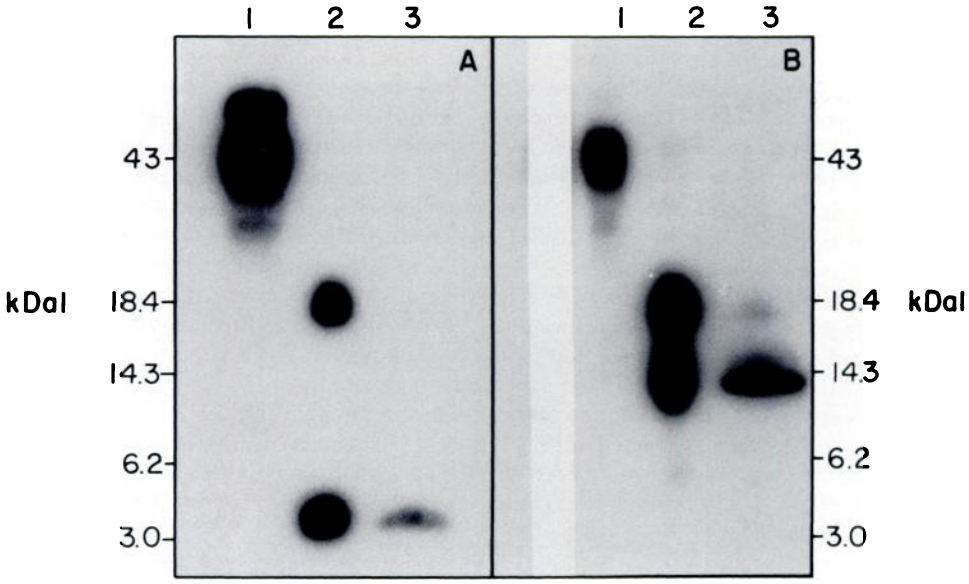

Fig. 5. Immunoaffinity extraction and Western blotting of plasma atriopeptin prohormone metabolites. Five rats were each given 3 μg of dAVP intravenously for 3 min before collecting blood. Blood was collected from the abdominal aorta into 0.1 part each of 110 mM sodium citrate, 50 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 1.25 × 10^6 units/ml aprotinin on ice. Plasma from this blood was immunoaffinity extracted with NTF affinity and AP affinity columns connected in series. Affinity extracts were eluted in acid, combined, lyophilized, and Western blotted with AP (blot A) and NTF (blot B) antisera. Lanes *1A* and *1B*, molecular weight markers (ovalbumin visualized); lanes *2A* and *2B*, 0.5 μg of ANF1-126-Arg-Arg fully cleaved by thrombin + 0.5 μg of uncleaved ANF1-126-Arg-Arg; lanes *3A* and *3B*, plasma from dAVP-treated rats (~100 ng for NTFir and 50 ng for APir).

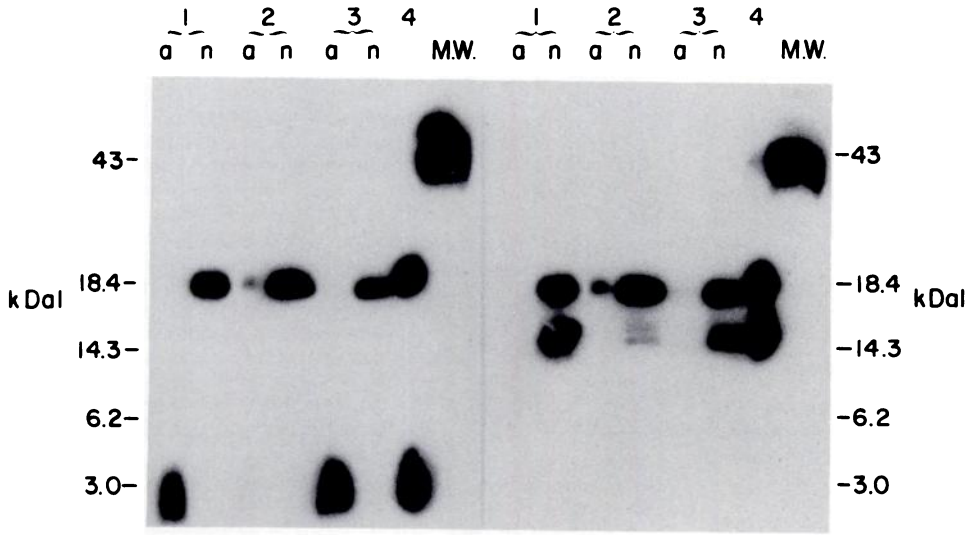

Fig. 6. Western blots of ANF1-126-Arg-Arg by isolated rat heart and by blood components. Samples were immunoaffinity extracted with NTF affinity and AP affinity columns connected in series. Affinity extracts were then separately eluted with acid, lyophilized, and Western blotted with AP (blot A) and NTF (blot B). AP affinity extracts (*a*) and NTF affinity extracts (*n*) were run on parallel lanes. Lanes *1a,n*, 15 μg of ANF1-126-Arg-Arg in 15 ml Krebs of buffer reperfused for 20 min through and isolated reperfused rat heart; lanes *2a,n*, 5 μg of ANF1-126-Arg-Arg incubated with 5 ml of rat plasma containing 1.5 mg/ml EDTA for 15 min at 37°; lanes *3a,n*, 5 μg of ANF1-126-Arg-Arg incubated with 5 ml of rat serum containing 1.5 mg/ml EDTA for 15 min at 37°; lanes *4A* and *4B*, 0.5 μg of thrombin-cleaved ANF1-126-Arg-Arg + 0.5 μg of uncleaved ANF1-126-Arg-Arg. *M.W.*, molecular weight standards.

in the absence of exogenous prohormone) yielded only a faint 3-kDa APir band, a faint 14-kDa NTFir band, and no intact prohormone (not shown). Incubation of exogenous prohormone with control reperfusate did not result in prohormone processing (data not shown).

Metabolism of exogenous prohormone by blood constituents. Processing of the ANF1-126-Arg-Arg substrate in blood and blood elements was also examined. Ten μg of ANF1-126-Arg-Arg were incubated for 15 min at 37° with 5 ml of plasma (1.5 mg/ml EDTA) or 5 ml of serum (EDTA added after preparation), after which protease inhibitors were added. The samples were extracted on consecutive AP and NTF affinity columns and Western blotting. Only a small amount of processing occurred following incubation in plasma (Fig. 6, A and B, lanes 2*a* and 2*n*), whereas nearly half of the prohormone was processed in serum (Fig. 6, lanes 3*a* and 3*n*). The NTFir and APir metabolites from the serum proteolysis comigrated with the metabolites generated both by reperfusion of prohormone through the rat heart (Fig. 6, lanes 1*a* and 1*n*) and by cleavage of prohormone with thrombin (Fig. 6, lanes *4*).

Discussion

The storage form for AP in the rat has been established to be the 126-amino acid prohormone, ANF1-126 (10). We have previously reported that the major circulating form of atriopeptin is the 28-amino acid C-terminal peptide AP28 (13) and that isolated perfused rabbit hearts release only low molecular weight AP as judged by gel filtration and bioassay (15). Others have confirmed that AP28 is the circulating species (25) and that AP28 is the species released by the isolated perfused rat heart (26). Since little, if any, low molecular weight AP is stored in the atria, the prohormone must be enzymatically cleaved to yield AP28, either during or shortly after release from the myocyte. Several investigators have demonstrated that basal release of AP from atrial myocytes in culture consists only of prohormone (16, 17) and have suggested that processing of the prohormone is a post-secretory event. Unfortunately, the form of AP released from cultured myocytes under stimulated (as opposed to basal) conditions has not been identified.

To study AP prohormone processing, we have developed an RIA which recognizes intact prohormone and the 98-amino acid NTF but which does not recognize the biologically active C-terminal APs. NTFir was detected in the plasma of anesthetized Sprague-Dawley rats and was found to be higher than the corresponding APir. Both plasma NTFir and plasma APir were increased in response to the administration of the potent vasoconstriction, dAVP. This was consistent with our hypothesis that the C-terminal APs and N-terminal metabolites of the prohormone were released together from the atria. Plasma NTFir remained elevated long after plasma APir had returned to normal, suggesting that those metabolites detected by the NTF-RIA had a longer half-life than the APs. We have confirmed this by studying the half-life of AP24 and recombinant NTF in rats.[2]

Acetic acid extracts of rat atria contained high levels of NTFir consistent with levels of atrial APir. Serial dilutions of both plasma and atrial extracts caused parallel displacements of [^{125}I]-N-Tyr-NTF48-67 in the NTF-RIA as compared to NTF48-67 (data not shown). Western blot analysis of both acetic acid and SDS extracts of rat atria showed that only a single NTFir band is present and that this band comigrates with the 128-amino acid recombinant prohormone construct, ANF1-126-Arg-Arg. Of interest was the appearance of a 6-kDa APir band in the acetic acid atrial extract which was not detected by the NTF blot. This probably represents an acid hydrolysis artifact since no such band was seen when the cold powdered atria were boiled in SDS-PAGE sample buffer.

We have used thrombin-cleaved ANF1-126-Arg-Arg as a Western blot standard for processed prohormone since the resulting NTF metabolite is identical to the first 98 amino acids of ANF1-126 and since the C-terminal thrombin metabolite, AP28-Arg-Arg, comigrates with authentic AP28 on 15% SDS-PAGE gels. N-Terminal sequencing and amino acid analysis of thrombin-cleaved ANF1-126 (purified from rat atria) and the recombinant prohormone ANF1-126-Arg-Arg has confirmed the specificity of this cleavage at the Arg^{98}-Ser^{99} bond (data not shown). Plasma from dAVP-treated rats was extracted with AP and NTF immunoaffinity columns, and the extracts were Western blotted. A single APir band was detected which comigrated with AP28, consistent with our earlier report

[2] N. Katsube, D. Schwartz, and P. Needleman, submitted for publication.

of AP28 as the major circulating AP in dAVP-treated rats (13). The major NTFir band on the blot comigrated with the 98-amino acid thrombin-generated standard. Only a trace amount of prohormone was detected with the NTF blot.

We used the isolated reperfused rat heart to see whether exogenous prohormone could be processed by the heart. Only faint 3-kDa APir and 14-kDa NTFir metabolites from endogenous prohormone were detected in the Western blot of control heart reperfusate. Intact prohormone was not detected. When exogenous prohormone was introduced into the reperfusion buffer, it was processed to metabolites which were indistinguishable from those detected in the control reperfusate and those generated by incubation of prohormone with serum or thrombin. This demonstrated that the isolated perfused heart can process both endogenous and exogenous prohormone and that the prohormone metabolites detected in plasma could be generated by the heart alone.

It has recently been reported that platelets (19) and serum (16) can process the AP prohormone *in vitro*. Our studies confirm that serum can process the prohormone, but we were unable to detect significant processing by plasma. It seems unlikely that a serum-generated enzyme is the physiologically relevant enzyme for prohormone processing *in vivo* since neither whole blood (data not shown) nor plasma processed the ANF1-126-Arg-Arg substrate. Such an enzyme need not be invoked to explain the processing of the prohormone since the heart appears to have sufficient enzymatic capacity. We conclude that the prohormone is processed immediately after release from the atrial myocytes *in vivo*. The location of the enzyme responsible for this is unclear.

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