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Research Article

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Synthesis and Secretion of an Atriopeptin-like Protein in Rat Kidney Cell Culture

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Abstract

The synthesis and secretion of an atriopeptin(AP)-like prohormone (AP126ir) has been demonstrated in rat neonatal renal cell cultures. AP126ir could be detected in the cellular extract and the medium from cultured kidney cells of neonatal and adult rats using an enzyme immunoassay specific for cardiac AP prohormone. On reverse-phase high-performance liquid chromatography, the AP obtained from the extract and the medium comigrated with cardiac AP prohormone. Incubation of the renal AP in the medium with thrombin resulted in the generation of a single low molecular mass peak which migrated with the cardiac carboxy-terminal 28-amino acid AP. Neonatal kidney cells pulsed with [^{35}S]methionine secreted radiolabeled AP126ir, which was detected by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis chromatography. Incubation of neonatal kidney cell cultures with the protein synthesis inhibitor cycloheximide resulted in a significant decrease in both the cellular and media AP. No decrease in cellular and media AP was detected when neonatal atrial cultures were treated with cycloheximide. These data demonstrate the de novo synthesis of an AP prohormone-like protein in neonatal rat kidney cultures. Furthermore, unlike the atria, kidney cells appear to secrete AP solely by constitutive means. In primary adult rat kidney cultures, most of AP126ir was detected in the cortical tubule fraction demonstrating that these cells secrete AP126ir in the adult rat kidney. We hypothesize that the renal AP may be important as an autocrine or paracrine regulator of renal function. (*J. Clin. Invest.* 1990. 87:208–212.). Key words: atrial natriuretic peptide • diuresis • factor • renal • sandwich enzyme-linked immunosorbent assay

Introduction

Atriopeptin (AP)[1] is synthesized in the mammalian atria and stored in atrial granules as a 126-amino acid prohormone (AP126) (1). Secretion and processing occur simultaneously since the circulating form of AP is the 28-amino acid carboxy-terminal fragment, AP28 (2). The kidney is a major target organ for AP28 and produces an increase in renal blood flow, glomerular filtration rate, and natriuresis (3). These effects are mediated by AP receptors in the kidney glomeruli and inner medulla (4). Furthermore, the kidney is a major clearance organ for AP28 via its proteolytic degradation by a neutral endopeptidase in the proximal tubule brush border (5, 6). Previous work in other laboratories have demonstrated the existence of a low molecular mass AP in kidney extracts and urine (7, 8). Since there is a high AP receptor binding capacity in the kidney, it was presumed that this renal AP was of blood origin. An amino-terminally elongated form of AP28 in human urine has been demonstrated, suggesting that this peptide was derived from renal origin (9). Our group has recently demonstrated AP immunoreactivity (APir) in the distal cortical nephron of rat kidneys (10). To further evaluate renal AP synthesis and its mechanism of secretion, we chose to study this peptide in neonatal and adult rat kidney cultures.

Methods

Cell culture. Kidneys were obtained from 4-d-old Sprague-Dawley rats. Whole kidneys were minced and cells were dissociated in 0.1% trypsin in Hanks' buffered saline without calcium and magnesium. The cells were centrifuged at 500 *g* for 10 min, and the pellet was resuspended in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated (2×10^6) in 35-mm diam wells (Becton, Dickinson & Co., Lincoln Park, NJ). Atrial and ventricular myocyte cell cultures were obtained from the atrial appendages and the ventricular apices of the neonatal rat heart, respectively. These cells were isolated in a manner identical to the above described kidney cultures and 1×10^6 cells were plated in 35-mm wells. The normal rat kidney (NRK) epithelial cell line (NRK-52E, 15th passage) was purchased from American Type Culture Collection, Rockville, MD. Rat mesangial cell cultures were generated according to the method of Lovett et al. (11). Kidneys from Sprague-Dawley rats (150 g) were removed and the isolated glomeruli were plated into wells and kept in RPMI 1640 medium, 5 mg/liter insulin, antibiotics, and 20% fetal calf serum. After 4 wk in culture, the remaining cell type consisted of mesangial cells. To generate papillary collecting duct and cortical tubular epithelial cell cultures, papillae and cortex were excised from kidneys of adult Sprague-Dawley rats (150–200 g), respectively. Cells from papilla and cortex were dissociated in 0.1% type I collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.1% BSA Krebs-Henseleit-buffer for 120 and 45 min, respectively. To the papillary cell suspension 2 vol of distilled water were added and allowed to stand for 3 min before plating. The cortical cell suspension was filtered through a nylon filter (52-μm mesh opening, Spectrum Medical, Los Angeles, CA) to remove glomeruli. The absence of glomerular contamination was confirmed by microscopic visualization. Cells, both cortical and papillary, were plated on type IV collagen (50 μg/ml)-coated wells at a concentration of 2.5×10^5 tubule fragments per well. Experiments were done after 1 wk when cultures reached confluence. For AP determination, cultures were incubated in defined media. The defined media consisted of DME/F12 (1:1) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM Hepes, 35 mg/liter human transferrin, 5 mg/liter bovine insulin, 20 μM ethanolamine, 25 μM sodium selenite, and 0.3% BSA, essentially globulin free (Sigma Chemi-

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1. *Abbreviations used in this paper:* AP, atriopeptin; EIA, enzyme immunoassay; ir, immunoreactivity; NRK, normal rat kidney.

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cal Co., St. Louis, MO). When cardiac AP prohormone (AP126) was incubated for 24 h in this media, no proteolysis was detected (unpublished observation). Cellular AP was extracted by boiling the cultures in 1 M acetic acid for 5 min followed by mechanical dissociation of the cells from the culture plates. After sonication and centrifugation (10,000 *g* for 3 min), the cellular supernatant was lyophilized and stored at −20°C.

The cellular protein content was determined with a protein assay (Bio-Rad Laboratories, Richmond, CA).

AP assay. AP was determined by using a competitive enzyme immunoassay (EIA) as described previously (12). In this assay, antibody (GP4) was used which had been raised in guinea pig against the rat AP sequence 103–126 (AP24). The cross-reactivity of this assay for rat AP126 was determined by the following procedure. The AP immunoreactivity (ir) was measured in a sample before and after incubation of AP126 with thrombin which selectively cleaves the AP prohormone between the amino acids Arg98 and Ser99 (13). The incubation with thrombin resulted in the total conversion of AP126 to AP28 which was confirmed by applying the sample to HPLC. Under these conditions we calculated a cross-reactivity of 33% for AP126 as compared to AP24 using the GP4 antibody.

For measurement of AP126, we developed a highly specific and sensitive noncompetitive EIA sandwich assay. This assay was performed as follows. A monoclonal antibody (MRW9C61) raised in mouse against AP24 (14) was initially bound to 96-well plastic plates (6106L10, Thomas Scientific, Swedesboro, NJ) by overnight incubation (100 μg/ml). Nonspecific binding to the wells was minimized by preincubation of wells with 1% gelatin. Standards and samples were dissolved in EIA buffer (12) containing 0.1% BSA (essentially globulin free) and incubated overnight at 4°C. Next the plates were washed and guinea pig antibody (GP58), which had been raised against AP48-66 (amino acid sequence 48–66 of the AP prohormone), was added. After a second wash, the synthetic peptide AP48-66, which had previously been coupled to acetylcholinesterase, was added. The amount of bound acetylcholinesterase was directly proportional to the amount of AP126 bound to GP58. Acetylcholinesterase activity was determined by the degradation of acetylthiocholine to acetate and thiocholine (12).

Characterization of kidney AP. AP of the cellular extract and medium were initially concentrated on a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) and eluted with 70% acetonitrile, 0.1% trifluoroacetic acid. Samples were then subjected to a C_{18} (4.6 × 250 mm) reverse-phase column (Separations Group, Vydak, Hesperia, CA). The column was run at a flow rate of 1.0 ml/min eluting with a linear gradient (25–60% acetonitrile, 0.1% trifluoroacetic acid) over 50 min. Fractions were collected every 2 min, lyophilized, and assayed for AP immunoreactivity.

Metabolic labeling with [^{35}S]methionine and immunoprecipitation of APir. Cultured neonatal kidney cells were incubated with methionine-free F12 medium containing 2 mCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL), 0.3% BSA, 10 μM thiorphan, and antibiotics overnight. Aliquots of the conditioned media without and with addition of 2.5 μM AP24 were used for immunoprecipitation with GP4 antibody after preclearance with normal rabbit serum. The immunoprecipitate was applied to a SDS gradient gel (gradient 17–27%, Geltech, Salem, OH).

Synthetic rat AP24 and thiorphan were obtained from Monsanto Co., St. Louis, MO. Reagents, if not specified otherwise, were purchased from Sigma Chemical Co. Rat AP126 was obtained from extracts of adult atria and purified by HPLC as described above.

Results

To validate the specificity of the AP126 EIA, standard curves using cardiac AP126 were generated in the presence and absence of AP24 and AP48-66. As seen in Fig. 1, the addition of 400 fmol per well of AP24 or 250 fmol per well of AP48-66 did not displace the prohormone standard curve. These results

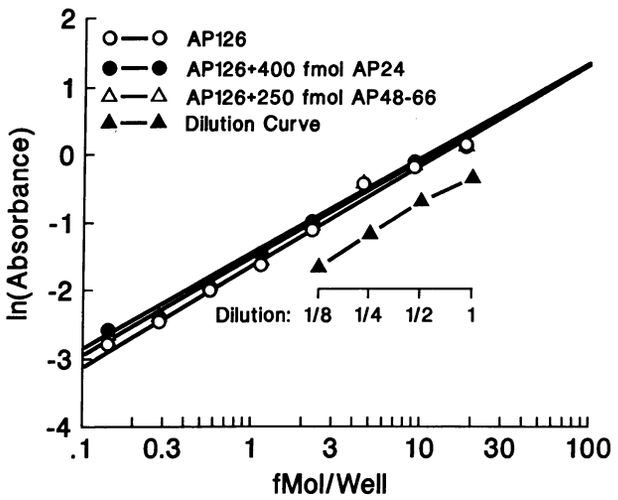

Figure 1. Specificity of the prohormone EIA. For this assay two antibodies were used: (*a*) monoclonal antibody (MRW9C61) directed against AP24 and (*b*) polyclonal antibody (GP58) directed against AP48-66. Standard curves of AP126 were generated in the absence (○) and presence of 400 fmol per well of AP24 (●) or 250 fmol per well of AP48-66 (△). The dilution curve was derived from secretion medium after incubation with renal cell culture for 24 h (▲).

confirm the specificity of this assay for AP126. The lower limit of detection was ~ 20 fmol/ml. The intra- and interassay coefficient of variability was determined to be 2.5% and 3.9%, respectively.

When neonatal kidney cells were incubated in secretion media for 24 h, we were able to detect ~ 200 fmol/ml (35-mm well) of APir in the prohormone-specific EIA. Dilution of this secretion media resulted in a curve that paralleled authentic rat atrial AP126 (Fig. 1). To evaluate the secretion of the AP-like peptide from these kidney cells with respect to time in culture, cells were extracted on days 1, 3, 4, 6, and 8 after plating. Before extraction cells were incubated in secretion medium for 24 h at 37°C. The cellular content of AP126-like immunoreactivity (AP126ir) increased from days 1 through 3 (4.2±0.1 to 6.8±0.3 fmol per well) after plating. On day 3, the cellular content of AP was maximum. 24 h later, the cellular content of AP126ir began to decline and reached a nadir on day 6 (2.5±0.2 fmol per well) (Fig. 2 *A*). The increase in the cellular content of AP126ir from days 1 to 3 may reflect the increase in cell number since these cultures became confluent on day 2. However, the decline in AP content after day 3 does not reflect cell number since the cultures were easily maintained confluent for at least up to 14 d. The AP126ir secreted into the media, with respect to cell age, paralleled the cellular AP126ir content. Maximal AP126ir secretion occurred on day 3 (204±10 fmol per well 24 h) (Fig. 2 *B*). Similar to the cellular content, secreted AP126ir began to decline on day 4 and reached its nadir on day 6 (22±2 fmol per well). Since AP28 undergoes degradation by proximal tubular brush border enzymes, and this degradation is inhibited by thiorphan, a neutral endopeptidase inhibitor (5), we evaluated whether thiorphan (10 μM) added to the media would increase the yield of AP126ir. No difference of the AP126ir concentration in the medium was found in the presence or absence of thiorphan as shown in Fig. 2 *B*. These data demonstrate that neonatal kidney cells secrete AP-like prohormone as detected in a prohormone-specific assay.

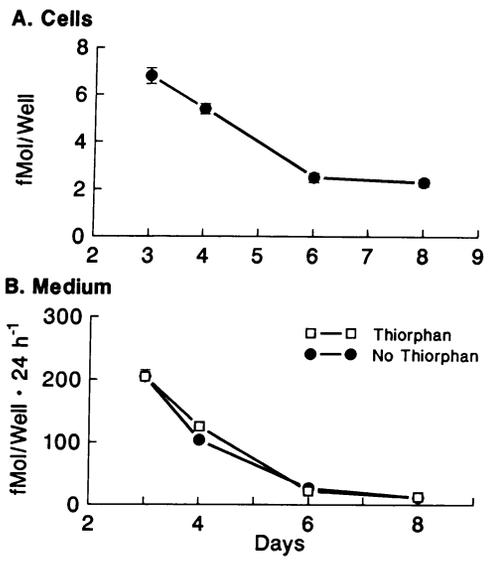

Figure 2. Time course of cellular and secreted AP126ir in neonatal renal cell culture. Cultures were grown in DME-10% fetal calf serum. To determine AP126ir, the cultures were incubated with secretion medium in the presence or absence of 10 μM thiorphan for 24 h and then extracted. AP126ir was determined in the cellular extract (*A*) and the secretion medium (*B*) with the prohormone specific assay. Values are expressed as femtomoles of AP126ir per well. Each time point is presented as mean±standard error of mean (n = 3).

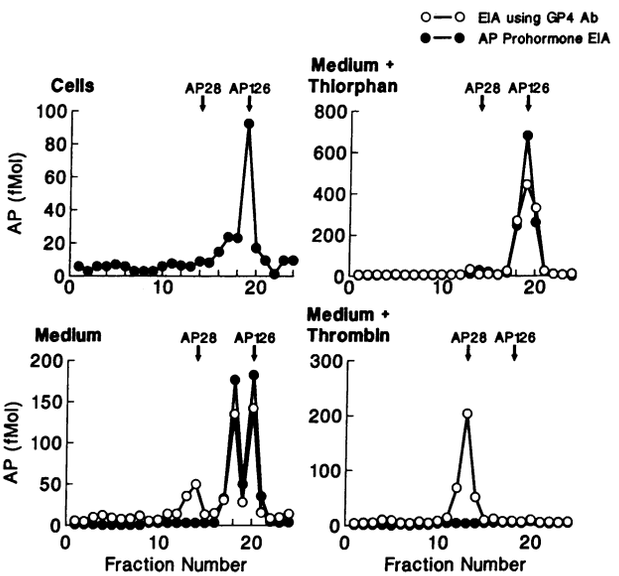

Figure 3. Chromatography of renal APir. 2-d neonatal kidney cell cultures were incubated with secretion medium for 24 h. Cellular extracts and media were characterized by reverse-phase HPLC. The retention times of AP standards are indicated by arrows. Fractions were assayed for APir with the EIA using antibody (GP4) raised against AP24 (○) and the prohormone specific EIA (●). Values were corrected for cross-reactivity. (*Top left*) cellular extract of renal cell culture; (*top right*) 24-h secretion medium with 10 μM thiorphan; (*bottom left*) 24-h secretion medium without thiorphan; (*bottom right*) 24-h secretion medium followed by a 1-h incubation with thrombin at 37°C.

The identity of this renal AP-like protein was further characterized by its migratory properties on reverse-phase HPLC. Chromatography of the cellular extract or the medium (Fig. 3) containing thiorphan showed a single peak coeluting with cardiac AP126. Deletion of thiorphan in the secretion medium resulted in the generation of two major peaks. Both peaks were detected by the prohormone-specific EIA, suggesting that one of the peaks may have been a truncated product of the AP-like peptide that maintained the recognition sites for the antibodies used in this assay. Incubation of the secretion medium with 10 μg/ml thrombin for 1 h at 37°C resulted in the generation of a single peak, detected by the carboxy-terminal directed EIA, eluting at the position of AP28 (Fig. 3). This peak could not be detected with the prohormone-specific assay. This demonstrates that the renal AP like protein, similar to atrial AP126, undergoes site-specific proteolysis by thrombin.

To evaluate the de novo synthesis of the AP-like peptide in neonatal kidney cell cultures, cells were incubated with radiolabeled methionine overnight before immunoprecipitation of the medium with GP4 antibody in the absence and presence of AP24. In lane *1* on SDS-PAGE chromatography (Fig. 4), the renal APir migrated with an apparent molecular mass of 12.5 kD. The specificity of this band for AP is demonstrated by its absence when an excess of AP24 was added to the sample before immunoprecipitation (lane *2*, Fig. 4). The demonstration of a specific high molecular mass radiolabeled band proves that renal APir is generated by de novo synthesis.

Atrial myocytes store AP in granules. Bloch demonstrated regulated secretion in atrial cell cultures and constitutive secretion in ventricular cell cultures by evaluating the amount of AP secreted versus the content of AP in cellular stores (15). Similar to the data of Bloch et al. (15), we determined media:cell AP ratios of 0.02 and 0.53 in atrial and ventricular cultures, respectively (Table I). Interestingly, the ratio in kidney cell cultures was determined to be 4.3, demonstrating little or no storage capacity for renal AP-like protein in these cells. To further evaluate the secretory pathway of the AP-like protein in neonatal kidney cells, we incubated neonatal atrial, ventricular, and kidney cell cultures with the protein synthesis inhibitor cycloheximide. We hypothesized that atrial cells, which have significant intracellular stores of AP, would not decrease their cellular content or secretion rate of AP in response to a short incubation with cycloheximide. Cell cultures were preincubated with cycloheximide (5 μM) for 1.5 h. After this preincubation period, cells were incubated for another 3.5 h with cycloheximide. The amount of AP126ir secreted within this 3.5-h period as well as the cellular AP126ir and total cellular protein content was determined. In both heart and kidney cell cultures, the total cellular protein content declined ~ 25% when compared to cultures which had not been incubated with cycloheximide (Fig. 5). Cellular APir decreased 60% and 47% in kidney and ventricle cells after treatment with cycloheximide, respectively.

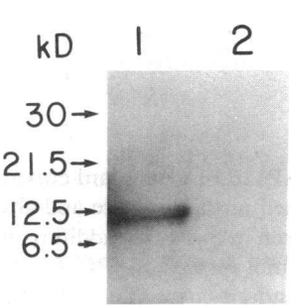

Figure 4. SDS-PAGE of biosynthetically labeled renal APir. Neonatal kidney cultures were incubated with [^{35}S]methionine (2 mCi) overnight, and aliquots of the conditioned media were immunoprecipitated with GP4 antibody in the absence (lane *1*) and presence (lane *2*) of 2.5 μM AP24. Immunoprecipitates were analyzed by SDS-PAGE gel chromatography.

Table I. Evaluation of the Cellular and Secreted Amount of AP126ir in Neonatal Atrial Myocyte, Ventricular Myocyte, and Kidney Day 2 Cell Cultures

Tissue source	AP in extract	AP in medium	Medium/Extract
	fmol/mg protein	*fmol/mg protein × h^{-1}*	*h^{-1}*
Atria	101,576±3,995	2,098±139	0.02
Ventricle	2,611±157	1,375±29	0.53
Kidney	5.7±0.4	24.7±0.3	4.35

APir was determined with the prohormone-specific assay. Each group is presented as mean±SEM (n = 4).

Table II. Secreted Amount of AP126ir in Adult Kidney Cultures

Tissue source	Result
	fmol/mg × protein × h^{-1}
Epithelial NRK cell line	1.1±0.1
Mesangial cell	Nondetectable
Cortical tubule	28.3±0.8
Papillary collecting duct	1.0±0.1

APir was determined with the AP prohormone-specific assay. Values are means±SEM (n = 3).

The amount of APir secreted in the presence of cycloheximide decreased by 88% and 81% in kidney and ventricular cell cultures, respectively. In contrast, cycloheximide had no effect on either the cellular or media AP levels from atrial cell cultures. We excluded that the decrease in cellular and secreted APir in ventricular and kidney cell cultures in response to cycloheximide was secondary to cell death, since the cells did not stain for trypan blue. Both the cellular content and the amount of secreted AP126ir decreased in renal cell culture after the incubation with cycloheximide, demonstrating that kidney cells secrete an AP-like peptide constitutively.

To evaluate the presence of the AP-like protein in adult rat kidney cell cultures, we examined NRK, primary papillary collecting duct, primary mesangial, and primary cortical tubular cell cultures. AP126ir was found in the conditioned medium of cultured NRK cells, papillary and cortical tubular cell cultures, whereas no AP126ir was detected in mesangial cultures (Table II). However, cortical tubular epithelial cells secreted 30 times more AP126ir when compared to other adult rat kidney cell cultures. To characterize the APir from adult cultures, conditioned medium of NRK and adult cortical cell cultures were applied to HPLC. APir eluted in a single peak with cardiac prohormone, demonstrating that the AP-like peptide from adult kidney cells is similar to the peptide from neonatal renal cell cultures (data not shown).

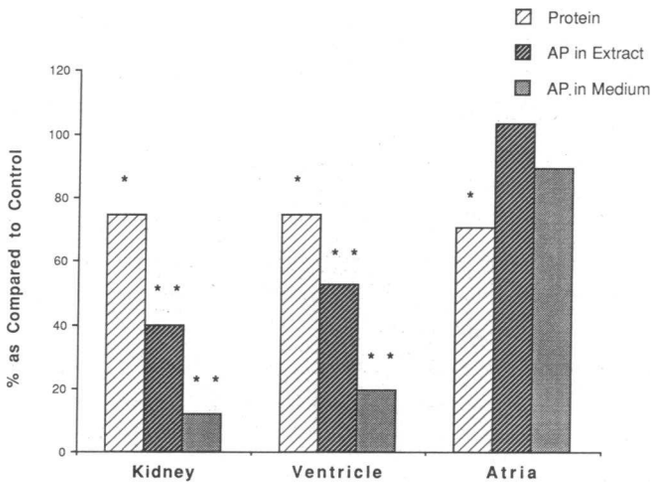

Figure 5. Effect of cycloheximide on cardiac and renal AP synthesis and secretion. On day 2, renal, ventricular, and atrial cell cultures were preincubated with 5 μM cycloheximide (CHX). After 1.5 h, the media were changed and the cells were incubated for another 3.5 h with secretion medium and CHX. Control cultures were incubated without CHX for the specified period of time. AP126ir was determined in the cellular extracts and the secretion media with the prohormone specific assay. Each group is presented as mean±SEM (n = 4). Significant differences of the protein and AP content in CHX-treated cultures versus control cultures (n = 4) were calculated with the unpaired Student t test (*$P < 0.05$, **$P < 0.001$).

Discussion

Previously our group has demonstrated an AP-like prohormone in nephrotic rat kidneys (10). Because the circulating form of AP has been identified to be AP28 (2), the carboxy-terminal fragment of AP126, the finding of an AP-like prohormone in rat kidneys suggested that the kidney synthesizes an AP-like peptide. To provide further evidence of renal AP-like peptide synthesis and to demonstrate the mechanism of AP secretion, we investigated AP126ir in primary neonatal renal and adult cell cultures and in the kidney cell line NRK.

Similar to our previous finding of AP126ir in nephrotic kidneys, we were able to demonstrate the existence of AP126ir in kidney cell cultures. This finding has been supported by the following issues. AP126ir was detected in the extract and medium of renal cell cultures with a highly specific EIA for cardiac AP prohormone. This EIA requires two antibodies raised against AP48-66 and AP24. Furthermore, AP was characterized in the renal extract and medium by reverse-phase HPLC and was found to comigrate with AP prohormone from heart extract. It has been previously reported that thrombin specifically cleaves AP prohormone between Arg98 and Ser99 generating AP28 (13). In our experiments, incubation of renal AP with thrombin resulted in the generation of a low molecular mass AP which comigrated with AP28 on HPLC. Although these results indicate the similarity of the renal AP-like peptide with cardiac AP126, we cannot rule out that the renal AP126ir may be different from the authentic atrial AP126.

To demonstrate the de novo synthesis of renal AP126ir, kidney cells were incubated with the radiolabeled amino acid methionine. This resulted in the labeling of a specific APir protein with an apparent molecular mass of 12.5 kD, thereby demonstrating the de novo synthesis of an AP-like peptide.

Once it was determined that neonatal kidney cells synthesize and secrete AP-like peptide, experiments were designed to determine whether this peptide was secreted via constitutive or regulated mechanisms. Comparison of the ratio of secreted to cellular AP126ir in renal and ventricular myocyte cultures showed a severalfold higher ratio as compared to atrial myocyte cell cultures. Incubation of the kidney cells with cyclohexi-

mide resulted in a large decrease in the cellular and secreted APir. Similar results were obtained for ventricular cell cultures. However, the stored and secreted AP from atrial myocyte cultures was unaffected by cycloheximide treatment. These studies of atrial and ventricular myocyte cells confirm the previous finding that atrial cells secrete AP by a regulated pathway whereas secretion in ventricular cells occurs by a constitutive mechanism (15). Furthermore, these results indicate that renal cells in culture secrete an AP126-like peptide in a constitutive manner.

Our finding that this AP-like peptide is not stored in renal cells may explain why AP-like prohormone has not previously been detected in kidney extracts from normal rats (8). These kidney extracts contained a considerable amount of low molecular mass AP. In the heart, AP126 processing occurs during or after secretion from the atrial myocyte. If the processing of the renal AP-like prohormone is analogous to the events in the atria, the renal low molecular mass AP may be partially derived from renal AP synthesis.

Atrial cells store AP in granules and release AP immediately after agonist stimulation. Because secretion of AP126ir in kidney cells appears to be solely constitutive, we hypothesize that the AP126ir secretion in the kidney, as in the cardiac ventricle, will not respond acutely to agonist stimulation. However, AP126ir secretion may be regulated by chronicly applied stimuli that may result in the changes of synthetic cellular capabilities.

Similarly to neonatal kidney cell cultures, we could demonstrate AP126ir in primary adult kidney cell cultures and in the NRK cell line. Since most of the AP126ir was found in the cortical tubular fraction, cortical tubules may be the cell type responsible for synthesis of this renal AP-like peptide in the adult kidney.

The synthesis of AP in the heart (1), the release of AP from the atria into the circulation (16) and the diuretic action of AP in the kidney (3) has been well established. Beside this endocrine pathway of AP, we suggest the existence of a paracrine function of AP in the kidney. The role of this pathway in the physiology and pathophysiology of the kidney has to be evaluated in further studies.

Acknowledgments

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