

## ARTICLE

## Expression of Adrenomedullin and Proadrenomedullin N-terminal 20 Peptide in Human and Rat Prostate

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**SUMMARY** Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are two recently discovered hypotensive peptides translated from the same message transcript (preproAM mRNA). In this article we report the presence of AM, PAMP, and their mRNA in human and rat prostate and of AM receptor mRNA in rat prostate. PreproAM mRNA was found in the epithelium of normal human and rat prostate glands by in situ hybridization. In humans, it was mainly expressed in the basal cells. In rat, its expression was higher in the ducts than in the acini of all the prostate lobes. Immunocytochemistry identified a similar distribution pattern for AM compared with its mRNA but showed different locations for AM and PAMP immunoreactivity. The former was widespread in the epithelia, whereas the latter was almost exclusively found in neuroendocrine cells. In rat, Western blot analysis confirmed the presence of high levels of AM peptide in the ventral lobe and of its precursor in the ventral and dorsolateral lobes. Immunoreactivity for serotonin, chromogranin A, PAMP, and AM defined four subpopulations of prostate neuroendocrine-like cells in rat, a cell type that has not been previously described.

(*J Histochem Cytochem* 47:1167–1177, 1999)

**KEY WORDS**

adrenomedullin  
proadrenomedullin N-terminal  
20 peptide  
adrenomedullin receptor  
prostate  
immunocytochemistry  
in situ hybridization  
RT-PCR  
Western blot

Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are two recently identified peptides with pluripotent function (Kitamura et al. 1993a,b). Both are amidated peptides (52 and 20 amino acids, respectively) originated by post-translational enzymatic processing of a single 185-amino-acid precursor, preproAM (Kitamura et al. 1993b, 1994). Human and rat AM consist of 52 and 50 amino acids, respectively, and show a very high homology (Kitamura et al. 1993a; Sakata et al. 1993). Rat and human PAMP are almost identical in their 20 amino acid residues. The sequence of AM bears some homology with calcitonin gene-related peptide (CGRP) and amylin but not with PAMP. Recently, receptors for AM (AM-R) have been cloned and sequenced (Kapas et al. 1995), and binding sites for PAMP have been localized (Iwasaki et al. 1996).

AM and PAMP are expressed in a variety of normal mammalian cells and organs showing enhanced expression during certain pathological states. In the adrenals, heart, and other cardiovascular tissues, the highest levels of these peptides have been reported (for a review see Montuenga et al. 1998). AM-R has been localized by in situ hybridization in different tissues (Martínez et al. 1997b; Montuenga et al. 1997).

Initially, it was demonstrated that AM and PAMP were potent vasodilators, but a number of additional roles have recently been attributed to these peptides. For example, AM may act as an autocrine growth factor (Miller et al. 1996), an apoptosis survival factor (Kato et al. 1997), a neurotransmitter (Allen and Ferguson 1996), a bronchodilator (Kanazawa et al. 1995), and an antimicrobial agent (Walsh et al. 1996). AM has also been reported to control hormone secretion (Martínez et al. 1996) or renal homeostasis (Massart et al. 1996). PAMP appears to be involved in inhibition of neurotransmission (Shimosawa et al. 1995) and also acts as a bronchodilator (Kanazawa et al. 1995),

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Received for publication November 12, 1998; accepted March 30, 1999 (8A4825).

an antimicrobial agent (Walsh et al. 1996), and a growth suppressor (Ando et al. 1997).

There are no studies on the expression of AM, AM-R and PAMP in the male urogenital tract, although some physiological reports on the effect of AM in penile erection have recently been published (Champion et al. 1997). The human prostate consists of compound glands connected to the prostatic urethra and surrounded by a fibromuscular stroma. The ejaculatory ducts and the utriculus prostaticus are included among the glands, which are disposed concentrically around the urethra. Throughout the entire glandular epithelium of the human prostate, three cell types can be distinguished (Luke and Coffey 1994): basal or stem cells, with proliferative ability; secretory or principal cells, which secrete prostatic fluid; and neuroendocrine (NE) cells, which produce bioactive amines such as serotonin and neuropeptides (Luke and Coffey 1994). NE cells are scant and are scattered throughout the epithelium of the glands, the urethra, and the utriculus. It has been suggested that they might be involved in the regulation of the proliferation of their surrounding cells via secretion of paracrine factors (Di Sant'Agnese and Cockett 1996).

Despite some biochemical, embryological, histological, and functional differences compared to the human prostate (Price 1973), the rat prostate has been widely used as a model to study prostate biology and pathology (Pollard 1992; Nishi et al. 1996). In the rat, the prostate is closely related to the ampullary glands, the ejaculatory ducts, and the urethra (Jesick et al. 1982). The rat prostate is composed of glands disposed in one dorsal, two lateral, and two ventral lobes around the urethra (Jesick et al. 1982). Each dorsal and lateral gland consists of a proximal duct, from which originate many distal ducts or acini (Hayashi et al. 1991). Ventral glands have also a proximal duct, which branches out into intermediate ducts (Lee et al. 1990) which, in turn, branch further into acini. The proximal ducts of all lobes are connected to the urethra (Jesick et al. 1982). These connections and the urethra are immersed in a thick fibromuscular stroma, all constituting the urethral ring. However, in the ventral lobes, distal segments of the proximal ducts spread out of the urethral ring. Principal and basal cells are found in the rat prostate epithelium. Principal cells are secretory in nature and may proliferate, and basal cells are scarce and also can divide (English et al. 1987). To our knowledge, NE cells have not yet been described in rat prostate.

The objective of the present work was to study the expression of AM, AM-R, and PAMP in the different cell populations of human and rat prostate. The results reported here suggest that AM and PAMP play a relevant role in the physiology of rat and human prostate.

## Materials and Methods

### Tissue Samples and Processing

Control human prostates were obtained from autopsies of healthy young individuals (17 and 23 years old) killed in traffic accidents (kindly donated by Dr. Luis Santamaría; Department of Morphology, Autonomous University of Madrid, Spain). All tissue procurement protocols were approved by the relevant institutional committees. Prostates were fixed in 10% formalin, dehydrated in alcohols, and embedded in paraffin. Sections 3  $\mu\text{m}$  thick were obtained and placed on slides previously treated with Vectabond (Vector Laboratories; Burlingame, CA) for immunocytochemistry, and on ProbeOn Plus slides (Fisher Scientific; Pittsburgh, PA) for in situ hybridization (ISH). Some reverse-face sections were prepared to assess co-localization of immunoreactivity.

Adult Wistar rats from the colony kept at the University of Navarra were used. Animals were treated according to the ethical standards approved by our institution. Rats were anesthetized by inhalation of ether and decapitated. The urogenital tracts were exposed by abdominal incision and quickly removed. Prostates and the related structures (e.g., ampullary glands) were immersed in 10% formalin or in Bouin's fluid for 24 hr. After fixation, the organs were cut sagittally, dehydrated, and processed like the human tissues. Some rat prostates were immediately frozen in liquid  $\text{N}_2$  to carry out RT-PCR and Western blot techniques. Ventral and dorsolateral lobes were separately processed for Western blotting.

### Antibodies and Optimal Dilutions

Immunoreactivity for AM was demonstrated using a polyclonal antiserum (no. 2469) raised in rabbits to the synthetic peptide P072 ( $\text{AM}_{22-52}\text{AMIDE}$ ). This antiserum was obtained from consecutive bleeds of the rabbit from which the antiserum no. 2343 was obtained (Martínez et al. 1995) and shares its immune properties. Different dilutions of the AM antiserum were tested (from 1:200 to 1:600). The optimal dilution was 1:200 in human and rat prostates. The same antiserum was used for Western blotting at 1:1,000.

To localize PAMP, one polyclonal antiserum (no. 2463) was used at 1:1000 for rat, and 1:4000 for human tissues. This antiserum was raised to the C-terminal peptide P070 ( $\text{PAMP}_{\text{YY13-20}}\text{AMIDE}$ ), which consists of eight common amino acids for human and rat peptides. The antiserum has been previously characterized (Montuenga et al. 1997). The antiserum no. 2463 was also used for Western blotting at 1:800.

Two monoclonal antisera raised against human chromogranin A were used to detect this protein. One of them (Boehringer; Mannheim, Germany) was used at a concentration of 2  $\mu\text{g}/\text{ml}$  for human tissues and 15  $\mu\text{g}/\text{ml}$  for rat. The other (Novocastra Laboratories; Newcastle, UK) was used at 1:50 from the commercial stock. Serotonin was demonstrated with a polyclonal antiserum (Incstar; Stillwater, MN), at 1:200,000 in human tissues and 1:125,000 in rats.

### Immunocytochemistry

Sections were deparaffinized, rehydrated, and endogenous peroxidase was inhibited in a 3%  $\text{H}_2\text{O}_2$  solution in deion-

ized H<sub>2</sub>O for 10 min. A microwave pretreatment was applied for antigen retrieval. Sections were immersed in 0.01 M citric acid (Sigma Chemical; St Louis, MO) buffer, pH 6, and heated for 10 min at 750 W, followed by 10 min at 375 W. Then the slides were cooled in running water. Tissues were blocked with normal rabbit serum (1:20) when monoclonal antisera were used or with normal swine serum (1:20) when polyclonal antisera were applied. Then the tissues were incubated overnight with the specific antiserum at 4C. After washes with Tris-buffered saline (TBS), sections were incubated for 1 hr in 1:100 biotinylated swine (against rabbit Fc; Dako, Glostrup, Denmark) or rabbit (against mouse Fc; Dako) IgG, according to the antibody employed. Slides were treated with the avidin-biotin-peroxidase complex (ABC; Dako) diluted 1:100 for 1 hr. Peroxidase activity was detected with 3-3'-diaminobenzidine hydrochloride (DAB)-H<sub>2</sub>O<sub>2</sub> and slides were counterstained with Harris' hematoxylin, dehydrated, and mounted in DPX.

Absorption controls were used to test the specificity of the antisera. Solutions containing the optimal dilution of each specific antiserum preincubated with its respective peptide (P070, P072, serotonin; Sigma) at 10 nmol/ml were applied to the slides instead of the primary antiserum. The chromogranin A peptides were not available.

#### Immunocytochemical Double Staining

Sections were deparaffinized, rehydrated, and endogenous peroxidase was inhibited. They were also microwave-preheated as described above. Slides were incubated with a normal sera mixture (1:30 goat, 1:20 swine), and incubated at 4C overnight with the specific antisera (monoclonal and polyclonal) mixture. Then the slides were covered with biotinylated swine anti-rabbit antiserum (Dako), and goat anti-mouse antiserum (Dako), both diluted 1:100, for 1 hr. Tissues were treated with monoclonal alkaline phosphatase-anti-alkaline phosphatase (APAAP, mouse monoclonal; Dako) and ABC at 1:50 and 1:100, respectively, for 1 hr. Goat anti-mouse antiserum was applied again, followed by APAAP, for 10 min each. The alkaline phosphatase (AP) was revealed using naphthol AS-TR and New Fuchsin as substrate, which produced a red endproduct. Peroxidase was revealed with DAB and nickel enhancement to give a black endproduct (Montuenga et al. 1992). Slides were mounted in PBS-glycerol.

#### Western Blotting

Prostates were homogenized in a buffer containing 50 mM NaCl, 25 mM Tris-HCl (pH 8.1), 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 10 µg/ml leupeptin, 25 µg/ml aprotinin, and 10 µg/ml pepstatin. Tissues were then clarified by ultracentrifugation, and the final protein concentration was determined (BCA kit; Pierce, Rockford, IL). Protein extracts were diluted to an approximate protein concentration of 35 µg/50 µl, heated to 95C for 3 min, and loaded into the sample well. Tissue protein extracts were electrophoretically separated on a gradient 10–20% tricine SDS-PAGE gel (Novex; San Diego, CA), and run at 100 V for 2 hr under reducing (5% β-mercaptoethanol) conditions. Synthetic AM 0.5 ng or PAMP 5 ng was added to separate wells as positive

controls. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a polyvinylidene difluoride membrane (Immobilon PVDF; Millipore, Bedford, MA) at 30 V for 3 hr. The membranes were blocked overnight in 1% BSA-PBS, incubated for 1 hr in a 1:1000 dilution of rabbit anti-AM or 1:800 of rabbit anti-PAMP, washed three times in PBS, exposed to 1 × 10<sup>6</sup> cpm [<sup>125</sup>I]-protein A for 30 min at 4C, washed six times in PBS, dried, and autoradiographed overnight at –80C on Kodak XAR5 film.

Solutions containing each specific antiserum preincubated with its respective peptide (P070, P072) at 10 nmol/ml were applied to the membrane instead of the primary antiserum and served as the absorption control.

#### Riboprobes

An 831-bp cDNA encoding for a fragment of preproAM was obtained from human adrenal mRNA by RT-PCR using the following primers: 5'-TAC-CTG-GGT-TCG-CTC-GCC-TTC-CTA-3' and 5'-CTC-CGG-GGG-TCT-CAG-CAT-TCA-TTT-3'. The PCR product was sequenced to ensure homology with the published cDNA (Genbank accession number: D14874) (Kitamura et al. 1993b). The amplified cDNA was ligated in both sense and antisense orientation into the expression vector pcDNA3 (Invitrogen; San Diego, CA) at the EcoRI site, following the manufacturer's instructions. SP6 promoter was used for generation of sense and antisense riboprobes to avoid differences in the in vitro transcription rate and efficacy. The sense or antisense plasmid was linearized with BamHI and used as a template to synthesize antisense or sense transcript, respectively, using SP6 RNA polymerase. Probe transcription was carried out at 37C for 2 hr. One µg of DNA template, nucleotides (including digoxigenin-UTP), 80 U of SP6 RNA polymerase (all from Boehringer), and 40 U of RNase inhibitor (RNasin Ribonuclease Inhibitor; Promega, Madison, WI) were used. Riboprobes were precipitated with ethanol at –20C and resuspended in H<sub>2</sub>O treated with diethylpicrocarbonate (DEPC; Sigma) containing 40 U of RNasin.

#### In Situ Hybridization

The protocol followed was similar to that applied by Garcia et al. (1998). Sections of formalin-fixed human or rat prostate were deparaffinized, rehydrated, and permeabilized in 0.2% Triton-PBS for 15 min. Rat tissues were digested at 37C with a proteinase K solution (10 µg/ml) in 0.1 M Tris-50 mM EDTA, pH 8, for 30 min. Human tissues were microwave-preheated as explained for immunocytochemistry and then incubated with proteinase K (5 µg/ml) at 37C for 15 min. Digestions were stopped in 0.1 M glycine-PBS. Tissues were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8, washed in DEPC-H<sub>2</sub>O, and air-dried at room temperature.

Hybridization with antisense probe (50 ng/µl hybridization buffer) was performed overnight at 43C in a moist chamber. Three stringency washes were carried out and then the slides were treated with RNase A (20 µg/ml) at 37C for 15 min. Sections were incubated for 2 hr with 1:500 anti-digoxigenin antiserum labeled with AP (Boehringer). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl

phosphate (Boehringer) were used to visualize AP activity (Polak and Van Noorden 1987). Slides were mounted in glycerol-PBS.

Two types of negative controls were used: the application of (a) the sense probe (50 ng/ $\mu$ l) instead of the antisense, and (b) mixtures of labeled antisense probe (50 ng/ $\mu$ l) with increasing concentrations (from 50 to 200 ng/ $\mu$ l) of unlabeled antisense probe.

#### RNA Extraction and RT-PCR

Total rat prostate RNA was obtained with the Ultraspec RNA Kit (Biotecx; Houston, TX), according to the manufacturer's instructions. The RNA concentration was spectrophotometrically determined. RNA was retrotranscribed with M-MLV reverse transcriptase (Gibco BRL; Paisley, UK). The sets of primers employed to detect mRNA of AM and AM-R through the polymerase chain reaction (PCR) are shown in Table 1. PCR was performed as previously described (Montuenga et al. 1997), using a P-E Thermocycler-2,400 (Perkin-Elmer; Foster City, CA) at 94C for 15 sec, 55C for 15 sec, and 72C for 1 min, for 35 cycles. PCR products were run in 2% agarose gels and scanned with a Molecular Analyst Biorad Laboratories Machine (Biorad; Hercules, CA), equipped with the Windows Software for Biorad's Image Analyzer Systems 1.4. The images were transformed into a TIFF format for printing.

## Results

#### Immunocytochemistry in Human Prostate

In the human tissues, labeling for AM was found mainly in the basal cells of the glandular epithelium (Figure 1) and the utriculus. AM was also found in the entire epithelium of the urethra, ejaculatory ducts, and squamous glands. Some cells of the stroma, endothelial cells, and nerves were also stained. Labeling was absent in absorption controls (Figure 2).

Strong positivity for PAMP was found in scattered cells throughout the epithelium of the glands (Figure 3), the utriculus, and the urethra. Most of them were located in the utriculus. These cells were more numerous in the glands of the central zone of the prostate than in the glands localized in the peripheral zone. The cells were either dendritic or nondendritic. Double immunolabeling for PAMP and chromogranin A confirmed these cells as neuroendocrine (Figures 4A

and 4B). Immunostaining of reverse-face sections demonstrated that PAMP-labeled cells were also stained for serotonin. However, not all the serotonin- or chromogranin A-immunolabeled cells were positive for PAMP (Figure 4B). Absorption controls confirmed the specificity of the immunostaining reactions.

#### Immunocytochemistry in Rat Prostate

In sections from rat prostate fixed in formalin and incubated with the anti-AM antiserum, the results were as follows. In the lateral lobes, the immunoreactivity was localized in the apical cytoplasm and in perinuclear areas of the secretory cells of the acinar epithelium (Figure 5). In the ventral prostate acini, only some cells with apical nuclei exhibited AM reactivity. In the dorsal lobe, a granular pattern of staining was found in the principal cells of the acini and in the luminal desquamated cells. AM immunoreactivity in the epithelial cells of the proximal ducts of all the lobes and in the ventral intermediate ducts was more intense than that observed in the acini. AM was homogeneously distributed throughout the cytoplasm of these cells. A small population of very intensely marked cells was scattered throughout the epithelium of the proximal ducts of all the lobes, the urethra and, less prominently, the ventral acini. These cells were oval and sometimes presented fine cytoplasmic extensions (Figure 6). Epithelial cells of the ampullary glands, urethra, and ureters were homogeneously stained for AM. Epithelial cells of the ejaculatory ducts were also immunoreactive and exhibited a granular pattern localized mainly in the upper half of the cells. The endothelial cells, some neurons of the associated ganglia, and some stromal (mainly smooth muscle) cells situated around the ducts in the periurethral zone were also positive for AM.

Absorption controls resulted in quenching of all AM staining except for that found in the scattered cells strongly stained. When a threefold (with respect to the optimal) dilution of the primary antibody was used, only these cells remained labeled (Figure 6), and the absorption control resulted in a total absence of labeling.

Strong immunoreactivity for PAMP was found in some dispersed cells of the epithelium of the proximal ducts of the lateral (Figure 7A), dorsal and ventral lobes. These PAMP-positive cells were also present in the epithelium of the urethra and, less frequently, of the ventral and dorsal acini. The distribution and shape of these cells were similar to those of the scattered AM-immunoreactive cells. However, reverse-face sections demonstrated that AM- and PAMP-immunoreactive cells are two different subpopulations because the immunoreactivities for both peptides do not co-localize in the same cells (Figures 7A and 7B).

**Table 1** Sequences of the oligonucleotides used for RT-PCR

Adrenomedullin (expected size 291 bp in human; 282 bp in rat)	
Sense (250-270)	5'-AAG-AAG-TGG-AAT-AAG-TGG-GCT-3'
Antisense (521-540)	5'-TGT-GAA-CTG-GTA-GAT-CTG-GT-3'
Adrenomedullin receptor (expected size 793 bp)	
Sense (687-706)	5'-ACC-AAT-ACC-TCT-CCC-TCC-TG-3'
Antisense (1462-1479)	5'-TGG-CAT-CCC-CCT-CT(CG)-AAC-3'
Adrenomedullin receptor (expected size 185 bp)	
Sense (1295-1314)	5'-GCA-CTC-CAT-CAT-CAT-TAC-CA-3'
Antisense (1462-1479)	5'-TGG-CAT-CCC-CCT-CT(CG)-AAC-3'

**Figure 1** AM immunoreactivity in the basal zone of human prostate gland. Bar = 17  $\mu$ m.

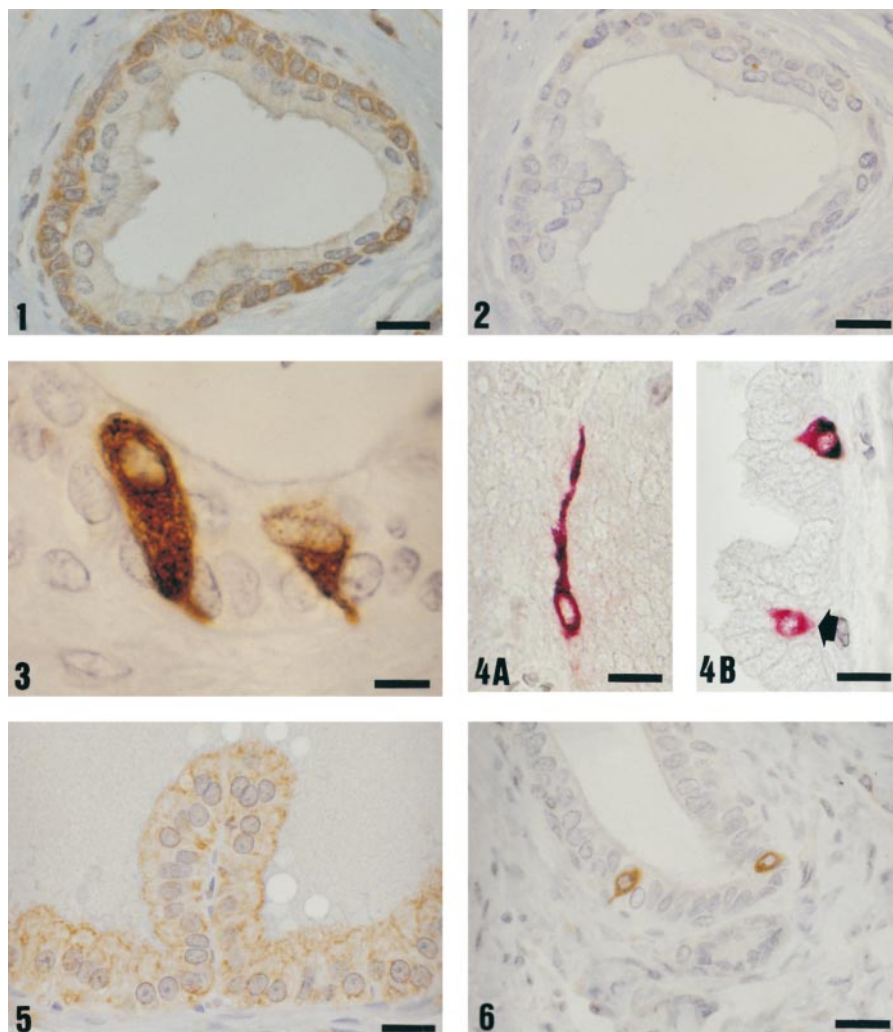
**Figure 2** Absorption control for AM in human prostate. Bar = 17  $\mu$ m.

**Figure 3** PAMP staining in human prostate. Bar = 7  $\mu$ m.

**Figure 4 (A,B)** Double immunocytochemistry for PAMP and chromogranin A in human prostate. A garnet color results from the co-localization of both red (for chromogranin A) and black (for PAMP) precipitates. There is a red cell positive only for chromogranin A (arrowhead). Bars = 17  $\mu$ m

**Figure 5** Rat lateral lobe acinus stained for AM. Immunolabeling is found in the epithelium. Bar = 17  $\mu$ m.

**Figure 6** AM-positive cells in a rat proximal duct draining the lateral lobe. Bar = 17  $\mu$ m.



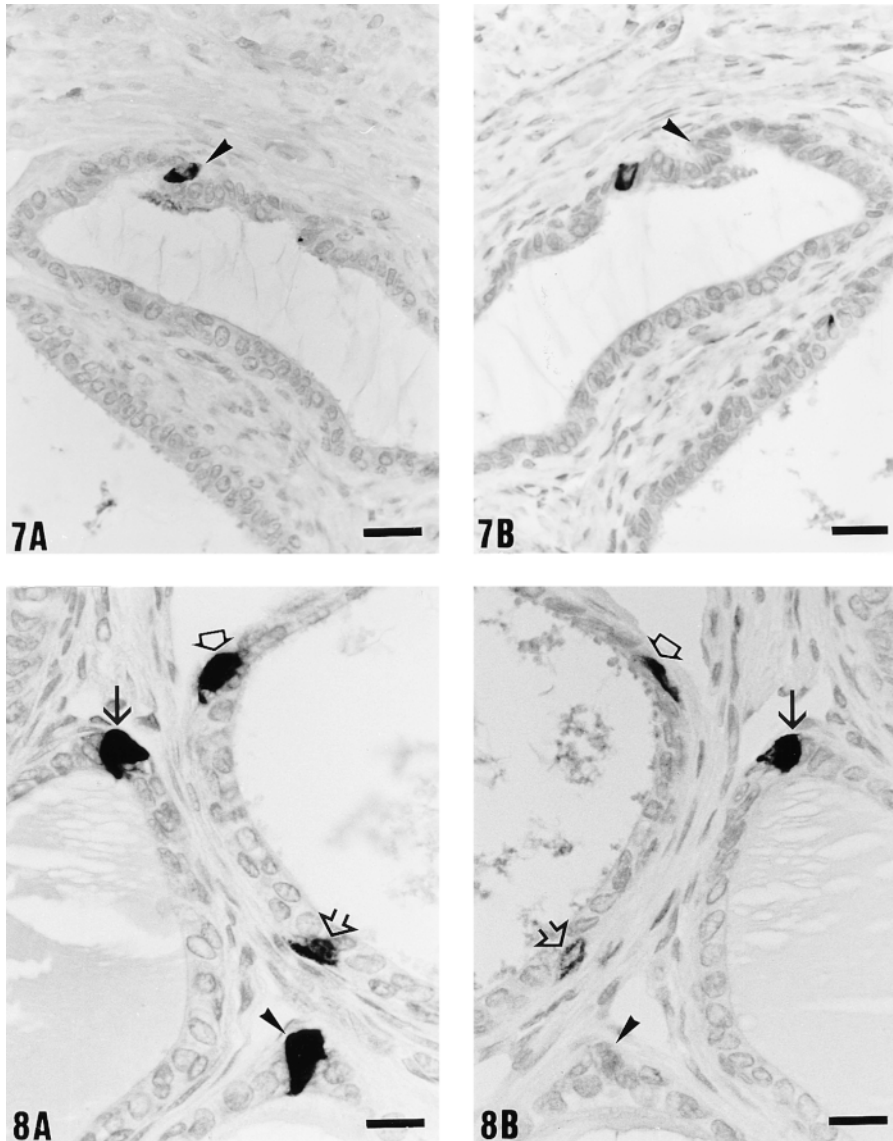
PAMP reactivity was also present in some ganglion perisomatic glial cells. Negative controls confirmed the specificity of the immunostaining for PAMP in all cases.

To characterize the nature of the AM- and PAMP-positive cells in the rat prostate, double immunocytochemistry (using anti-chromogranin A antisera) and immunocytochemistry in reverse-face sections (with anti-serotonin antiserum) was performed. A wide population of serotonin-positive cells was demonstrated dispersed in the epithelium of the urethra and the proximal ducts of all prostate lobes (Figure 8A). They were very rarely observed in the ventral acinar epithelium (i.e., one or two positive cells in the ventral lobes per sagittal section). The shape of these cells varied from oval to dendritic, the last resembling the typical NE cell type. Absorption controls resulted in lack of staining. In reverse-face sections, cells immunolabeled for PAMP were also serotonin-positive; however, not all the serotonin-positive cells were labeled for PAMP

(Figures 8A and 8B). There was no co-localization of AM and serotonin in the same cells. With two different antisera, the presence of scattered chromogranin A-positive cells was observed in the epithelium of the urethra and the proximal ducts of all the lobes. The chromogranin A-immunoreactive cells were less numerous than serotonin-positive cells. These cells appeared oval and sometimes exhibited cytoplasmic elongations. None of the chromogranin A-immunoreactive cells were simultaneously stained for AM or for PAMP, although a clear co-localization with serotonin was observed.

Fixation with Bouin's fluid enhanced the intensity of the immunoreactivity for all antibodies in the endocrine-like cells as compared to formalin. However, the rest of the cells labeled for AM were not stained in Bouin's-fixed tissues.

All the results described here were obtained in sections subjected to microwave treatment. The same immunoreactivities were observed in non-preheated rat



**Figure 7** Serial reverse-face sections of rat prostate lateral proximal ducts stained for (A) PAMP and (B) AM. Arrowheads point to the same cell in the epithelium. Bars = 20  $\mu$ m.

**Figure 8** Serial reverse-face sections of rat prostate dorsal proximal ducts stained for (A) serotonin and (B) PAMP. The same cells in both sections are marked with the same type of arrow. Note that the cell marked with arrowheads is positive for serotonin but not for PAMP. Bars = 12.5  $\mu$ m.

and human sections, with the antibodies used at a higher concentration. However, microwave preheating was indispensable to obtain reactivity for chromogranin A in rat prostate.

#### Western Blotting of Rat Prostate Protein Extracts

Total protein extracts from dorsolateral and ventral rat lobes were separately studied. Two immunoreactive bands for AM were found, one of approximately 14 kD and a second one, found only in the extracts from ventral prostate, of 6 kD (Figure 9A). The 6-kD band found in the ventral prostate co-migrates with the synthetic AM used as control (Figure 9A). Absorption control, with the same antigen used to raise the antibody, completely abolished the immunoreaction (Figure 9B). When the same protein extracts were run

and analyzed for PAMP immunoreaction, no bands were found (not shown).

#### In Situ Hybridization in Human and Rat Prostate

In human tissues, hybridization with the preproAM antisense riboprobe resulted in the staining of the basal cells in most of the glands (Figure 10) and in the utriculus. AM mRNA was also homogeneously distributed in the epithelial cells of the urethra, ejaculatory ducts, and glands exhibiting squamous epithelium. The stroma did not exhibit any staining. Incubations with mixtures of labeled and unlabeled antisense probes resulted in a decrease of the staining directly dependent on the proportion of unlabeled probe.

In rat prostate, hybridization with the antisense probe revealed the presence of AM mRNA in the epi-

thelial cells of the lateral acini, where the maximal labeling was found (Figures 11 and 12), in most cells of the dorsal acini (including desquamated cells), and in some ventral acinar epithelial cells (most of them with apical nuclei). Some neurons in the associated ganglia (Figure 13) and endothelial cells of most vessels were also stained. AM mRNA was also found in the epithelium of ampullary glands (Figure 15), proximal ducts of all prostate lobes, and ventral intermediate ducts, all showing strong positivity. Epithelial cells of ejaculatory ducts, urethra, and ureters were also labeled. Rat prostate incubated with mixtures of labeled and unlabeled antisense probes showed the same reduction of staining described for human prostate sections. In the tissues treated with the sense probe, labeling was absent (Figure 14).

In our hands, the optimal pretreatments for ISH in the prostate sections are as follows: (a) for human tissues, application of microwaves and proteinase K. Pretreatments with only proteinase K or only microwaves did not produce any staining; (b) in rat prostate, incubation with proteinase K was critical. Treatments with only microwaves or microwaves with proteinase K decreased the labeling in most zones. In both species, when sections were preincubated with only proteinase K, high background staining appeared in the stroma. This background was observed even when the sections were incubated with hybridization buffer alone. Nevertheless, this nonspecific staining did not appear in the sections of both species when microwave treatment was applied.

#### RT-PCR of Rat Prostate mRNA Extracts

In rat prostate, a 282-bp PCR product was detected after amplification with the human AM primers, demonstrating the presence of mRNA for preproAM (Figure 16A). Using two sets of primers to detect mRNA for AM-R, we also found the expected 185- and 793-bp PCR products (Figure 16B).

#### Discussion

Since its initial identification in a human pheochromocytoma, AM has been shown to be expressed in a variety of tissues (Ichiki et al. 1994; Satoh et al. 1995). In this work we demonstrate that AM and its gene-related peptide, PAMP, are also present in normal human and rat prostate, as determined by several analytical techniques. In rat prostate extracts, we have also detected by RT-PCR the expression of the AM-R mRNA.

AM is widely distributed in normal human and rat prostate, mainly in the epithelial compartment. Immunocytochemistry and ISH analysis show a parallel pattern of distribution for protein and mRNA.

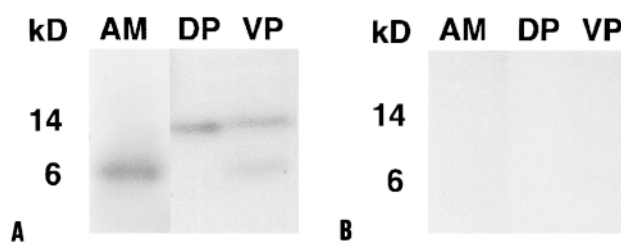
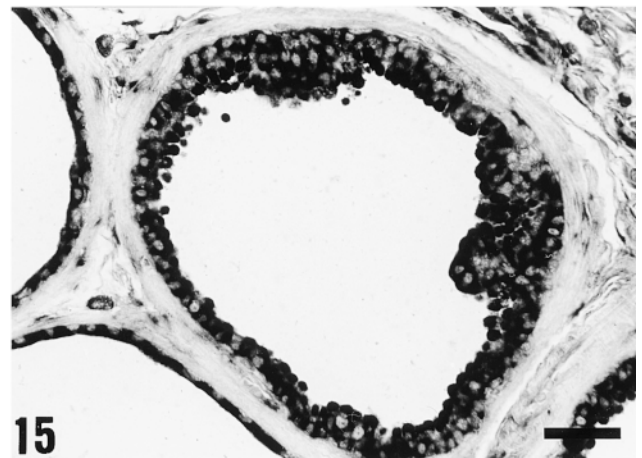
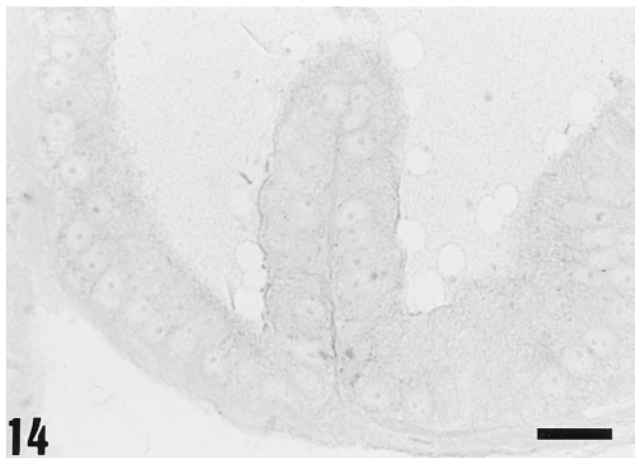
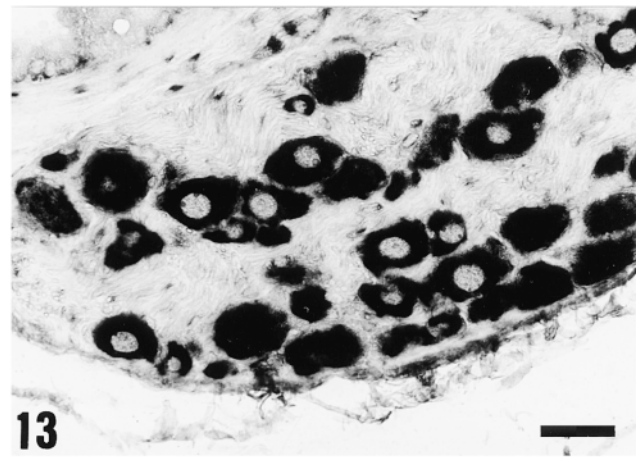
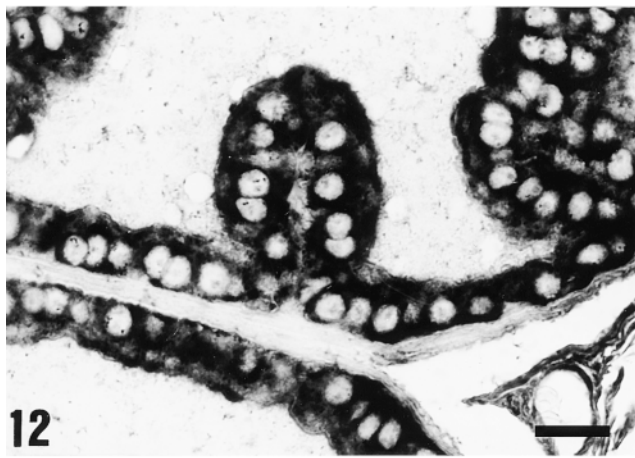
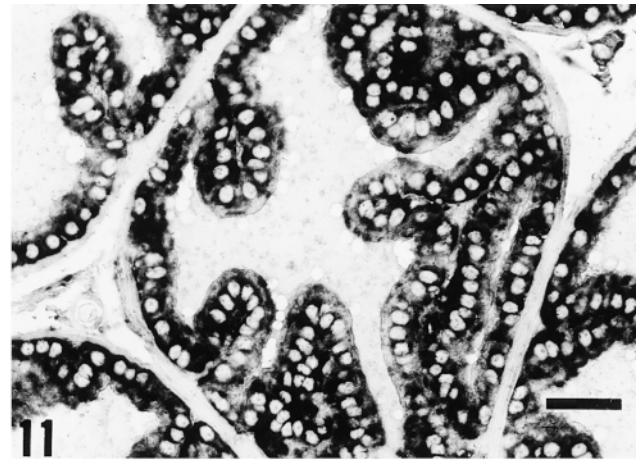
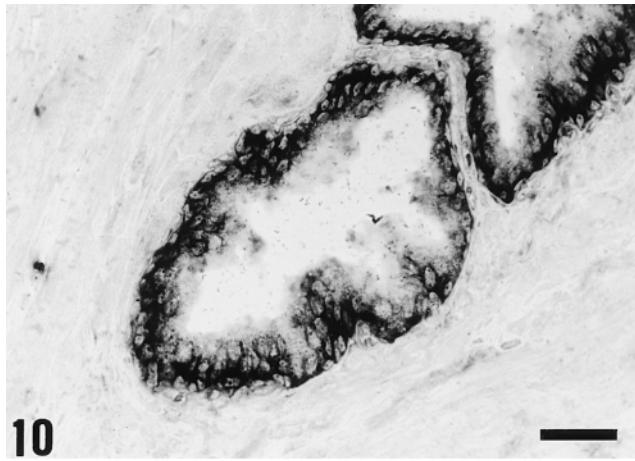


Figure 9 (A) Western blotting detecting AM (6-kD band) in rat ventral prostate and preproAM (14-kD band) in rat ventral and dorsolateral prostate. (B) Absorption control. VP, ventral prostate; DP, dorsolateral prostate.

In rat, the expression of AM depends on the zone of the gland, with the lateral lobes having the greatest amounts of mRNA and protein. The proximal ducts of all prostate lobes exhibit a higher expression than the acini, as revealed by immunocytochemistry and ISH. Lobe-specific differences in AM expression are also shown by Western blotting. A single 14-kD band, which may correspond to one of the AM precursors, is found for dorsolateral lobes, whereas bands of 14- and 6-kD (the latter corresponding to fully processed AM) are observed in the ventral lobe. This difference suggests a region-specific post-translational processing of the precursor preproAM in the ventral lobe vs dorsolateral prostate or the rapid secretion or degradation of this 6-kD entity in dorsolateral prostate. It is noteworthy that the protein extracts of dorsolateral or ventral lobes used to perform the Western blotting did not include the same portion of ducts; the dorsolateral prostate included mainly acini, whereas the extracts of ventral lobes included not only acini but also intermediate ducts and a segment of proximal ducts. On the basis of the results of the Western blot study, it is likely that the AM-like material detected by immunocytochemistry in rat acini is mainly preproAM, which could represent a storage form. However, the more intense immunolabeling detected in proximal ducts of all the lobes and in the ventral intermediate ducts could represent both preproAM and fully processed AM.

AM is involved in cell growth (Miller et al. 1996), epithelial repair (Martinez et al. 1997a,b), and antimicrobial activities (Walsh et al. 1996), and all these activities may be relevant in prostate physiology. It has been suggested that AM may act as an autocrine/paracrine growth factor (Miller et al. 1996; Montuenga et al. 1997). The widespread distribution of AM in the prostate and the presence in the same tissue of the mRNA for the AM-R (at least in rat) are consistent with the possibility that is a regulator of prostate cell growth.

AM also acts in the control of smooth muscle contraction, therefore regulating vasodilatation (Heaton et al. 1995) and bronchodilatation (Kanazawa et al.



**Figure 10** ISH localizing the preproAM mRNA in the human normal prostate. Bar = 40  $\mu$ m.

**Figure 11** Detection of mRNA for preproAM in the lateral lobe acinar epithelium of the rat. Bar = 40  $\mu$ m.

**Figure 12** Detail of Figure 13 showing strong labeling in the cytoplasm but not in the nuclei of the prostate cells. Bar = 20  $\mu$ m.

**Figure 13** ISH using the antisense riboprobe in a nerve ganglion associated with the rat prostate. Bar = 40  $\mu$ m.

**Figure 14** ISH performed with the sense riboprobe of preproAM mRNA in rat prostate as a negative control. Labeling is absent from the lateral lobe acinar epithelium. Bar = 20  $\mu$ m.

**Figure 15** PreproAM mRNA detected in the rat ampullary gland epithelium. Bar = 40  $\mu$ m.



1995). In the rat, AM is especially abundant in the epithelial cells of all the proximal ducts and the ventral intermediate ducts, which are surrounded by several layers of smooth muscle cells. The smooth muscle layer associated with the acinar epithelial cells, which express lower levels of AM, is less conspicuous (Nemeth and Lee 1996). In human prostate, smooth muscle cells are disposed around the basal compartment (Luke and Coffey 1994) of the glandular epithelium; AM is particularly abundant in the basal cells. Furthermore, the contraction of the smooth muscle cells in human prostate is believed to be related to the control of glandular fluid secretion (Luke and Coffey 1994). The spatial localization of AM in the prostate suggests that AM could be involved in the control of prostate secretion via regulation of duct muscle tone.

As shown by our results, PAMP expression in prostate is restricted to NE (in human) or NE-like (in rat) cells. PAMP immunoreactivity was undetectable by Western blotting. This is not surprising, given the small number of cells bearing this immunoreactivity as assessed by immunohistochemistry in this region of the rat prostate.

In humans, PAMP-positive cells represent a subgroup of serotonin-positive cells that belong to the larger population of NE cells expressing chromogranin A. Until now, the presence of PAMP in endocrine cells has not been described except for the adrenals and the pituitary (Montuenga et al. 1998). It is remarkable that the distribution of AM and PAMP immunoreactivities does not coincide either in human or in rat prostate. These differences in the localization of expression of both peptides suggest either a specific post-translational processing of the preproAM or an alternative mRNA splicing mechanism, depending on the cell type. A similar specific regulation of the expression of AM and PAMP has been suggested for other organs, such as the pituitary (Montuenga et al. 1998).

PAMP acts as a vasodilator, a function shared with AM (Kitamura et al. 1993a, 1994), and inhibits neurotransmission (Shimosawa et al. 1995). However, its presence in human NE cells leads to the suggestion that PAMP may also regulate local cell function as a paracrine peptide. Some regulatory substances have been previously described in NE cells of human prostate, including serotonin, thyroid-stimulating hormone (TSH), somatostatin (Luke and Coffey 1994), calcitonin, CGRP, and katacalcin (Di Sant'Agnes et al. 1989).

In rats, the study of the location and possible function of NE cells has received very little attention. Angelsen et al. (1997) did not find chromogranin A in the lobes of the rat prostate complex using different techniques. This is in agreement with our findings in which NE-like cells are virtually nonexistent in this area.

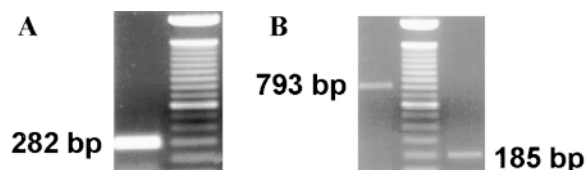


Figure 16 Demonstration by RT-PCR of mRNA for (A) preproAM and (B) AM-R in extracts of rat prostate.

However, our results very clearly show that NE-like cells are present in the prostate, although they are mostly restricted to prostate urethra and periurethral ducts.

We have described four subpopulations of NE-like cells in rat prostate according to their immunoreactive properties. The four groups of cells show respectively immunoreactivity for: (a) serotonin, (b) serotonin and PAMP, (c) serotonin and chromogranin A, and (d) AM. Two main differences were found between rat and human NE cells. First, in rats, chromogranin A-positive cells do not coincide with those positive for PAMP. In fact, chromogranin A cells are very scarce, being only a subpopulation of the serotonin-immunoreactive cells. Second, there is a subpopulation of NE-like cells positive for AM in rat (not in human) but they are not immunostained for chromogranin A. These results are consistent with the two antisera we have employed for detecting chromogranin A. Similarly to humans, PAMP-positive cells in rat prostate also constitute a subpopulation of serotonin-positive cells. The co-localization of PAMP and serotonin in the same cells implies that both factors are connected in rat and human prostate and that they may therefore contribute to regulation of the same physiological phenomena.

Some observations related to the techniques merit discussion. We have verified that Bouin's fixation is better than formalin fixation to detect NE cells (but not to demonstrate AM immunoreactivity in the other epithelial cells) of the rat prostate. This is in accordance with previous observations reported in human prostate for other substances (Abrahamsson et al. 1987). Microwave pretreatment enhanced the staining of all the antibodies used in our study, both after Bouin's and after formalin fixation. Using this treatment for ISH in human tissues, a decrease in background and signal enhancement (when used with proteinase K) were found. For rat prostate, microwave heating decreases the background but also decreases the staining. Therefore, the use of the optimal pretreatment has great importance for the in situ detection of AM, PAMP, other related peptides, and their respective mRNAs.

In summary, AM, AM-R, and PAMP have been found in several cell types of rat and human prostate.

Their distributions and the functions attributed to these regulatory peptides suggest that they may be relevant in normal biology of the prostate and could be the target for new studies aimed to assess their involvement in prostate physiology and pathology.

#### Acknowledgments

Supported by the Spanish Ministry of Education (DGICYT PB93-0711).

We thank Dr L. Santamaría (Department of Morphology, Universidad Autónoma de Madrid) for providing the normal human prostate tissues. We also thank I. Ordoqui and A. Urbiola for technical assistance, J. Lecanda and Dr J.A. Rodríguez for help in the performance of RT-PCR, and Drs M. García and C. García-Corchón for technical advice on ISH.

#### Note Added in Proof

After this article went to press, a new report appeared demonstrating that AM message is abundant in rat prostate epithelial cells and that it is regulated by androgens [Pewitt EB, Haleem R, Wang Z (1999) *Endocrinology* 140:2382–2386].

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