Journal of Neuroendocrinology, 2000, Vol. 12, 607–617

Expression of Proadrenomedullin Derived Peptides in the Mammalian Pituitary: Co-Localization of Follicle Stimulating Hormone and Proadrenomedullin N-20 Terminal Peptide-Like Peptide in the Same Secretory Granules of the Gonadotropes

L. M. Montuenga,* M. A. Burrell,* M. Garayoa,* D. Llopiz,* M. Vos,† T. Moody,† D. García-Ros,* A. Martínez,† A. C. Villaro,* T. Elsasser‡ and F. Cuttitta†

*Department of Histology and Pathology, School of Medicine, University of Navarra, Pamplona, Spain.
†Department of Cell and Cancer Biology, DCS, NCI, NIH, Rockville Pike, Bethesda, MD, USA and
‡US Department of Agriculture, Agriculture Research Service, Beltsville, MD, USA.

Key words: adrenomedullin, proadrenomedullin, *N*-terminal 20 peptide, gonadotropes, pituitary.

Abstract

Expression of proadrenomedullin-derived peptides in the rat, cow and human pituitary was studied by a variety of techniques. Immunocytochemical detection showed a widespread expression of adrenomedullin peptide in the adenohypophysis and the neural lobe, with low expression in the intermediate pituitary. Proadrenomedullin N-20 terminal peptide (PAMP)-immunoreactivity was also present in the anterior pituitary but showed a more marked heterogeneous distribution, with cells going from very strong to negative immunostaining. Lower levels of PAMP were found in the neural lobe. Interestingly, the distribution of adrenomedullin and PAMP immunoreactivity in the anterior pituitary did not completely overlap. In the present study, we concentrated our efforts to determine which cell type of the adenohypophysis expresses PAMP. Paraffin and semithin serial sections immunostained for PAMP and the classical pituitary hormones revealed that a subpopulation of the gonadotropes expresses high levels of PAMP-immunoreactive material. Ultrastructural analysis clearly showed PAMP-immunoreactivity in the follicle stimulating hormone (FSH)-containing large secretory granules of the gonadotropes, suggesting simultaneous secretion of PAMP and FSH by this cell type. Three mouse adenohypophysis-derived cell lines (AtT20, GH3, and αT3-1 derived from corticotropes, lacto/somatotropes and gonadotropes, respectively) were also analysed and showed expression of both proadrenomedullin-derived peptides and their mRNA. Functional studies in these three cell lines showed that neither adrenomedullin nor PAMP was able to stimulate cAMP production in our experimental conditions. Taken together, our results support that proadrenomedullin derived peptides are expressed in the pituitary in cell-specific and not overlapping patterns, that could be explained by differences in postranslational processing. Our data showing costorage of PAMP and FSH in the same secretory granules open a way by which PAMP could be involved in the control of reproductive physiology in a coordinated manner with FSH.

Adrenomedullin (AM) is a 52 amino-acid amidated peptide that was isolated from pheochromocytoma extracts and was shown to be a very potent hypotensive molecule in several mammalian models (1). Most of the reported biological effects of adrenomedullin are mediated through the stimulation of adenylate cyclases. AM cDNA has been cloned in several mammals, including human, rat, cow, pig and mouse (2–6). A second amidated peptide, also a hypotensive molecule, proadrenomedullin N-20 terminal peptide (PAMP) was predicted from the amino-acid sequence of its precursor molecule preproadrenomedullin (7), and has indeed been detected in plasma of several species, although in lower

Correspondence to: L. M. Montuenga, Department of Histology and Pathology, School of Medicine, University of Navarra. C/Irunlarrea 1 Pamplona 31080, Spain (e-mail: lmontuenga@unav.es).

concentrations than its gene-related peptide adrenomedullin (8, 9).

Although most of the investigative groups working on AM are concentrated on this peptide's role in cardiovascular function, it has now been realized that adrenomedullin may have much broader pluripotent properties, such as the modulation of bronchodilation (10), aldosterone (11) or insulin (12) secretion, or glomerular filtration and fractional sodium reabsorption (13). More recently, the involvement of AM in the regulation of growth and proliferation has also been reported for several cell types in which it has either a stimulatory (fibroblastic or tumoral cells) or inhibitory (mesangial cells) effect (14). Broad agreement has now been achieved towards the concept that adrenomedullin is a multifunctional peptide, similar in activity to tumour growth factor-β.

There are several reported findings that support the hypothesis of adrenomedullin playing a potential regulatory role in pituitary physiology. A number of investigative approaches have already shown expression of adrenomedullin or PAMP in mammalian pituitary. Radioimmunoassays of protein extracts of rat, human and pig pituitary have shown the presence of moderate to high amounts of immunoreactive adrenomedullin (15–17). The immunoreactive material from human pituitary extracts coeluted with synthetic human AM 1–52 in reverse phase high-performance liquid chromatography (18). Northern blot analyses have also demonstrated adrenomedullin expression in several areas of human brain, including the anterior pituitary (18). Washimine *et al.* (19) reported the presence of cells immunoreactive for adrenomedullin in the human and porcine anterior pituitary, but did not observe any immunoreactivity in the rat hypophysis. Their report was a review of a variety of organs in several species, and was not aimed particularly to study the expression of adrenomedullin and PAMP in the pituitary at the cellular level. Several physiological studies have suggested that exogenously added adrenomedullin can affect the secretion of pituitary hormones either *in vitro* or *in vivo*. Adrenomedullin suppressed basal and corticotropin releasing factor (CRF)-induced adrenocorticotropic hormone (ACTH) release from preparations of dispersed rat pituitary cells (20). Although the *in vivo* studies have not shown yet a clear picture of the regulatory mechanism involved, the reported data suggest that, in addition to its cardiovascular and fluid regulatory actions, adrenomedullin may be an important hormone involved in the regulation of pituitary/adrenal function in mammals (21, 22). Finally, a specific regulation of adrenomedullin expression in the pituitary gland was already suggested by the fact that in the promoter region of the gene coding for mouse adrenomedullin, one Pit-1 binding motif was identified, among other several putative regulatory sequences (6).

Although there are biochemical and functional data supporting a role for adrenomedullin in pituitary biology, until now, very little is known about the actual endocrine cell type within the hypophysis that produces and probably secretes adrenomedullin. This lack of information prompted us to investigate the distribution of adrenomedullin and PAMP expression in the mammalian pituitary. We were also particularly intrigued by the fact that the ratios of reported PAMP/adrenomedullin concentrations measured by radioimmunoassay (RIA) are higher in the pituitary than in any other organ, except for the adrenals. This suggested differential regulation of the expression of PAMP in the pituitary compared to other tissues. In the present study, we show that there is differential regulation in the expression of both peptides in separate cell types within the mammalian hypophysis.

Materials and methods

Tissues

Male and female adult Fisher rats were used to obtain the pituitary. Rats were killed by inhalation of CO_2 and rapidly decapitated. Pituitaries and adrenal glands were also obtained from normal cows and pigs used for other investigations approved by several USDA experimental protocols. These animals were euthanized by captive bolt and exsanguinated. Adrenal gland and pituitary were obtained and immediately placed either into liquid nitrogen or fixative. Human specimens for histological analysis came from the Department of Pathology of the University Hospital of Navarra and were obtained under an approved tissue procurement protocol. Tissues for histological analysis were fixed by immersion either in Bouin's fluid or in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 h and then washed in 70% ethanol previously to dehydration and paraffin embedding. Sections of 3–5 μm in thickness were obtained. We performed a pilot study for the optimal time of fixation with Bouin's solution for our immunocytochemical detection in rat pituitary and we concluded that 12 h gave the best results.

Eight rat pituitaries were processed for plastic embedding. Fixation was achieved by immersion in 0.1% glutaraldehyde plus 4% paraformaldehyde in phosphate buffer (0.01 M; 0.15 M ClNa; pH 7.4) at 4 °C for 2.5 h. Following a 16-h wash at 4 °C, pituitary fragments were dehydrated through ethanol and propylene oxide and embedded in Epon 812 (23). Semithin sections (1 μm thick) were stained with methylene blue in borate buffer. By inspection of semithin sections, suitable fields were selected for ultrathin sections which were stained with uranyl acetate and lead citrate and observed under an electron microscope. Immunocytochemical techniques were carried out on semithin and ultrathin sections as detailed below.

Cell lines

Three cell lines derived from mouse pituitary adenomas were used in the present study. GH3 cells are derived from somatotrope/lactotrope adenoma and were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS). AtT20 are corticotrope derived cells and were grown in DMEM medium supplemented with 10% FCS. αT3-1 cells were derived from a transgenic mouse in which SV-40 T large antigen is expressed driven by the human glycoprotein hormone α-subunit promotor giving raise to gonadotrope-derived adenomas. This cell line has the biological profiles of an immortalized gonadotrope but does not express the mRNA for the follicle stimulating hormone (FSH) or luteinizing hormone (LH) β subunits. αT3-1 cells were grown in DMEM medium supplemented with 10% FCS. GH3 and AtT20 cells were a generous gift of Dr Betty Eipper (Department of Neuroscience, Johns Hopkins University); αT3-1 were donated by Dr Pamella Mellon (Department of Reproductive Medicine, University of California at San Diego). Cells were harvested from T175 flasks when they were at 80% confluence and one day after feeding with fresh medium. Total RNA was obtained by the guanidine isothiocyanate and caesium chloride protocol. For immunocytochemical staining, harvested cells were fixed either in Bouin's fluid or in 4% paraformaldehyde in PBS, washed in PBS, and preembedded in 2% low melting agarose (FMC Bioproducts, Rockland, ME, USA). The solidified agarose pellets were embedded in paraffin for sectioning.

Antibodies

Previously reported well-characterized rabbit anti-AM 22–52 (#2469) or anti-PAMP 10–20 (#2463) antisera were used to localize adrenomedullin and PAMP immunoreactivity in pituitary tissues and cell lines (24, 25). The antibody against adrenomedullin was directed to AM 22–52 amide (*C*-terminal). The antibody against PAMP was directed to PAMPYY 13–20 amide (*C*-terminal). Antibodies against rat prolactin (rPRL-IC-5), rat growth

hormone (rGH-IC-1), rat thyroid stimulating hormone (THS-IC-1), rat FSH (rFSH-IC-1), rat LH (rLH-IC-1), and human ACTH (hACTH-IC-2) were generously donated by the National Hormone and Pituitary Program of NIDDK, NIH, USA.

Immunocytochemical technique

For the immunocytochemical localization of adrenomedullin and PAMP in paraffin sections, the avidin-biotin complex (ABC) technique was employed. Optimal dilution for anti-AM 22–52 was 1 : 1000, whereas the anti-PAMP 10–20 was used at 1 : 700. Following overnight treatment with the primary antiserum, sections were incubated with biotinylated goat antirabbit antiserum (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 200. The sections were then treated with the avidin-biotin peroxidase complex (Vector Laboratories) diluted 1 : 100 in PBS. Peroxidase activity was demonstrated using diaminobenzidine/H_2O_2 as chromogen and substrate, respectively. Finally, the sections were lightly counterstained with Gills' haematoxylin. In some cases, the reaction was intensified with metallic nickel (26). The intensifying developing reagent was made by mixing a solution of 50 mg of DAB in 50 ml of deionized H_2O with a second solution made up by adding 2.5 g of ammonium nickel sulphate (di-Ammonium nickel (II) sulphate 6-hydrate; BDH Laboratory Supplies, UK), 200 mg of β-D-glucose (Sigma Chemical Co., St Louis, MO, USA), 40 mg of ammonium chloride (Sigma) and 30 mg of glucose oxidase (Sigma), in 50 ml of acetate buffer (acetic acid 0.1 M pH 6).

Solid-phase absorption controls were carried out by preincubation (overnight at 4 °C) of the primary antiserum with the equivalent peptide previously adsorbed to the wall of a polystyrene tube at a concentration of 10 μg/ml. Another negative control included the use of nonimmune swine serum as first layer and gave no immunocytochemical reaction.

Immunocytochemistry at the semithin level was carried out as follows. After removing the plastic with a saturated solution of NaOH in methanol (27), semithin sections were rinsed in absolute ethanol, rehydrated in a graded series of ethanol, and washed twice in TRIS-HCl buffered saline. The avidin-biotin peroxidase complexes method was identical to that described above, but microwave pretreatment was used and the incubation time for the primary antisera was increased to 48 h. Microwave pretreatment consisted in a first 15 min incubation of the sections in a citrate buffer 0.01 M, pH 6.0 at full microwave heating power, followed by a second 15 min incubation in the same buffer at half power and a 5-min incubation without heating. Finally, the sections were washed during 10 min in running water. The reaction was intensified with nickel as described above.

Ultrastructural immunolabelling

For the ultrastructural studies, the colloidal-gold technique of Merighi *et al.* (28) was applied to thin sections mounted on nickel grids. Non-specific binding sites were blocked with 10% goat immunoglobulins in a buffer [Gold Buffer, GB, Tris-Buffered Saline with 1% bovine serum albumin (BSA) and 0.05% sodium azide] that was also used for the remaining steps. Thin sections were incubated overnight at 4 °C with the primary rabbit antiserum (1 : 200) in GB. Subsequent steps, all at room temperature, included rinses in GB, incubation with the gold-labelled secondary antiserum (goat antirabbit immunoglobulins conjugated to 20-nm gold particles, Biocell Research Laboratories, Cardiff, UK) for 1 h, rinses in GB and distilled water, and double staining with uranyl acetate and lead citrate. The grids were observed with a transmission electron microscope.

In situ *hybridization*

Detection of the mRNA of adrenomedullin was performed using *in situ* hybridization. A 831-bp adrenomedullin cDNA was obtained from human adrenals 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 amplified product was ligated in both sense and antisense orientation into the expression vector pcDNA3, containing SP6 and T7 promoters and used to generate riboprobes. In order to avoid differences in the *in vitro* translation rate and efficacy, the same promoter was used for the generation of sense and antisense riboprobes. The sense or antisense plasmids were linearized with *Not*I and were used as templates to synthesize digoxigenin-labelled sense or antisense RNA transcripts driven through T7 promoter. Hybridization was performed in a moist chamber at 46 °C for 20 h in a 20 μl volume containing the probe. After stringency washes, visualization of digoxigenin was performed using Digoxigenin detection kit (Boehringer Mannheim, Indianapolis, IN, USA). Sense probes were used as controls and rendered negative results in most of the cases; in some other cases minor staining was observed in the endocrine cells that were more strongly stained with the antisense probe, probably due to incomplete linearization of the plasmid combined with leakage of the unused promotor.

Western blot

To extract the proteins, the cell samples were immersed in cold 2X tricine sample buffer (with 8% SDS, NOVEX, San Diego, CA, USA) containing 1 mM final concentration of each of the following protease inhibitors: pefabloc (Centerchem Inc., Stamford, CT, USA), bestatin and phosphoramidon (Sigma). The cells were then homogenized, sonicated, clarified by ultracentrifugation, and the final protein concentration determined (BCA kit, Pierce, Rockford, IL, USA). Protein extracts were diluted to an approximate protein concentration of 35 μg/50 μl, heated to 95 °C for 3 min and loaded into the sample well. Cell or tissue protein extracts were electrophoretically separated on a gradient 10–20% tricine, SDS-PAGE gel (NOVEX), and run at 100 V for 2 h under reducing 0,5% β-mercaptoethanol conditions. One ng of synthetic adrenomedullin or 5 ng of PAMP was added to a separate well as a positive control. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a polyvinyldifluoride membrane (Immobilon PVDF, Millipore, Bedford, MA, USA) at 30 V for 3 h. The membrane was blocked overnight in 1% BSA-PBS, incubated for 1 h in 1 : 1000 dilution of rabbit anti-AM 22–52 (#2469) or anti-PAMP 10–20 (# 2463), washed three times in PBS, exposed to 1×10^6 cpm ^{125}I-Protein A for 30 min at 4 °C, washed 6 times in PBS, dried and autoradiographed overnight at −80 °C on Kodak XAR5 film. Specificity control consisted of a duplicate membrane incubated in antigen-preabsorbed (10 nmol/ml) antiserum.

Northern blot

Equal amounts of total RNA (20 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde, transferred to 'Nytran' filters (Scleicher and Schuell, Keene, NH, USA), UV cross-linked and baked for 3 h. Ethidium bromide (33 μg/ml) was included in the gel to visualize the position of ribosomal RNAs by ultraviolet illumination after electrophoresis. Hybridization was performed using a 0.55-kb *Eco*RI fragment of mouse adrenomedullin (plasmid pcRIIAM). Blots were hybridized with ^{32}P-labelled (3000 Ci/mmol, DuPont, Boston, MA, USA) random-primed probes at 65 °C, washed and exposed at −70 °C using a DuPont Lightning Plus intensifying screen (DuPont).

cAMP assay

Cyclic AMP was assayed by RIA. Cells in 24-well plates were resuspended in SIT medium (3×10^{-8} M Se_2O_3; insulin, 5 μg/ml; transferrin, 10 μg/ml) containing 1% BSA, 1 mg/ml bacitracin, and 100 μM isobutyl-methyl-xanthine. Adrenomedullin or PAMP was added and after 5 min the reaction terminated by adding equal volumes of ethanol (0.5 ml). The cAMP RIA kit was purchased from NEN (Boston, MA, USA).

Results

Immunocytochemistry of mammalian pituitary

Immunoreactivity for adrenomedullin and PAMP was found in human, rat, and cow anterior pituitary (Fig. 1A–D for rat; E–F for cow; G–H for human). Immunocytochemical staining using anti-adrenomedullin antibody showed an extensive and generally homogeneous reactivity in a high proportion of endocrine cells throughout the rat anterior pituitary, together with an also homogeneous staining in the neurohypophysis adjacent to the anterior lobe (Fig. 1A). The cells of the intermediate pituitary were generally not immunostained, with the only exception of some nuclei of endocrine cells that were positive and the flat epithelial cells that separate the anterior from the intermediate pituitary that were strongly immunoreactive (Fig. 1A). Nuclear staining was sometimes also observed in endocrine cells of the anterior lobe (not shown). Immunocytochemical results using polyclonal antibodies against PAMP showed staining in the anterior but not

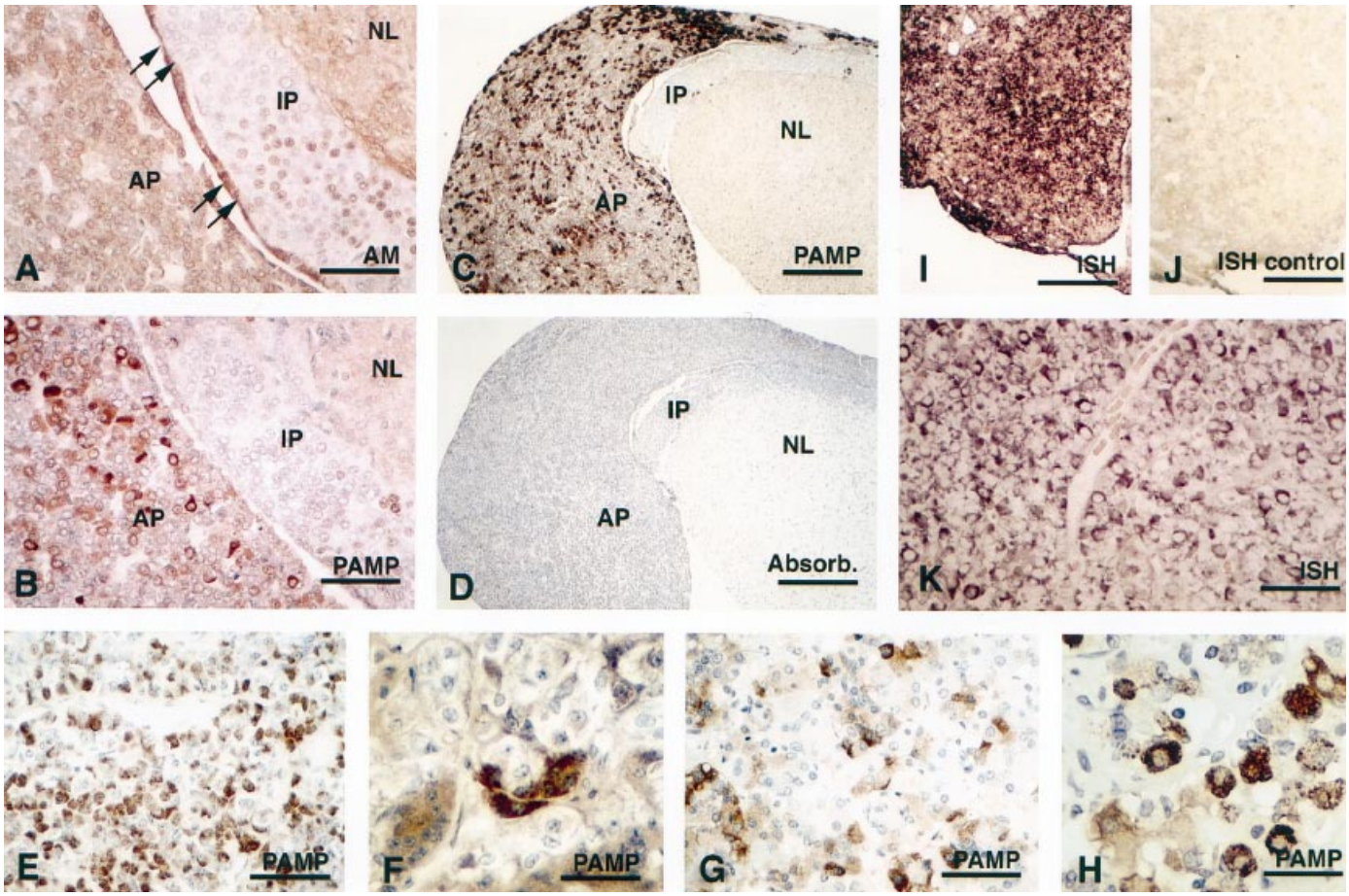

FIG. 1. Serial reverse-face sections of rat adenohypophysis immunostained for adrenomedullin (AM) (A) and proadrenomedullin N-20 terminal peptide (PAMP) (B). Note the non overlapping staining pattern. Panoramic view of the rat pituitary immunostained with an antibody against PAMP (C) and the same antibody absorbed with excess peptide (D). Cow anterior pituitary immunostained for PAMP (E, F). Three types of cells concerning immunostaining are clearly seen, especially at higher magnification (F), with high, intermediate and undetectable staining. Similar pattern of staining for PAMP is found in human anterior pituitary (G, H). (I–J) Representative examples of sections of rat pituitary on which *in situ* hybridization technique was performed to demonstrate AM mRNA, using an antisense (I) or a sense (J) probe. (K) Showing a detail of a region similar to (I), to illustrate heterogeneity of the staining among the adenohypophysis cells. (A, B, E, G, K × 360 bar = 40 μm; C, D × 70 bar = 200 μm; F, H × 720 bar = 20 μm; I, J × 150 bar = 100 μm.

in the intermediate lobe (Fig. 1B,C). The posterior lobe showed a homogeneous weak immunoreaction for PAMP, that was readily absorbed in the controls (Fig. 1B–D). In the anterior lobe, three types of cells could be distinguished according to the immunostaining pattern for PAMP: some cells were strongly positive, a second subpopulation showed a positive immunoreaction of lower intensity and a third subpopulation of cells were completely negative for this antibody (Fig. 1B,C,E–H). In the rat, the cells expressing high levels of PAMP-immunoreactive material tended to be concentrated in the periphery of the anterior lobe, but especially near the intermediate pituitary at the tuberal portion (Fig. 1C). Image analysis with relative measurements (area percentage of immunostained cells *vs* stained plus non stained cells) showed significant differences ($P < 0.002$) between sexes in the immunocytochemical detection of PAMP in rat pituitary, the males showing higher immunostained area percentage than the females. A similar pattern of distribution of adrenomedullin and PAMP immunostaining was observed when human and cow pituitary were stained, although in these species the heterogeneity in the intensity of adrenomedullin was higher as compared to the rat. Western blot of human pituitary extracts (Fig. 2) gave adrenomedullin and PAMP immunoreactive bands, mainly corresponding to the proadrenomedullin molecule (approximately 22 kDa) and a processed species (aproximately 17 kDa). In 2 out of 10 cases of human pituitary studied (shown in Fig. 2), a 6-kDa species, very likely corresponding to completely processed adrenomedullin could be detected in the western blot using anti-adrenomedullin antibodies. Similar bands could be detected in western blots of cow pituitary (data not shown).

Expression of adrenomedullin mRNA in the rat pituitary

In situ hybridization experiments revealed the presence of the mRNA for adrenomedullin in a variety of endocrine cells of the rat anterior pituitary (Fig. 1I–K). Not all the cells of the adenohypophysis showed detectable levels of adrenomedullin mRNA by this technique, and some degree of heterogeneity of the intensity of staining suggested differences in the levels

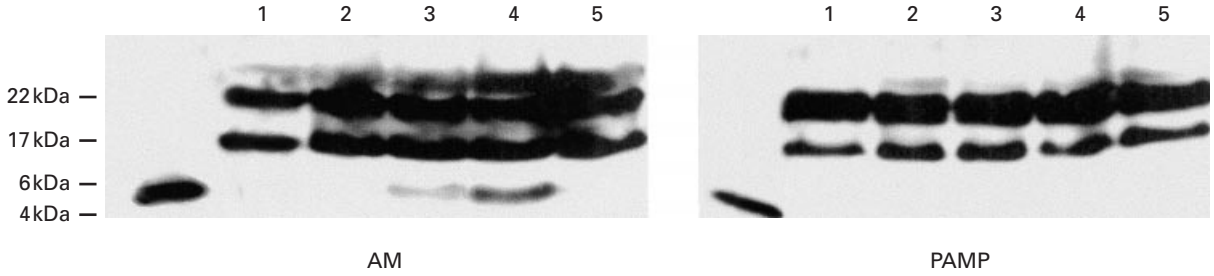

FIG. 2. Western blot analysis of proadrenomedullin N-20 terminal peptide (PAMP) and adrenomedullin (AM) in protein extracts of human pituitary. A total of 10 samples were studied, of which five representative ones are included. The 22 kDa and 17 kDa bands that are detected in most of the lanes very likely correspond to the preproAM precursor and/or intermediate species in the propeptide processing. Only in samples 3 and 4 a 6-kDa signal is found, corresponding to processed AM.

of expression among the positive cell types. Intermediate and neural hypophysis were negative for adrenomedullin mRNA expression. Sense probes were used as controls and rendered negative results. The *in vitro* transcription product from an empty plasmid was also used as a negative control and gave completely negative staining.

Expression of adrenomedullin in mouse pituitary cell lines

In order to compare the expression of adrenomedullin and PAMP in different pituitary cell types, mouse cell lines αT3-1, AtT-20 and GH3 (derived from mouse gonadotrope-, corticotrope- and somatotrope/lactotrope-adenomas), were used. Western blot (Fig. 3A), northern blot (Fig. 3B) and immunocytochemical (Fig. 4) analysis were carried out. Western blot analysis for adrenomedullin usually did not show immunoreactive bands for a 6-kDa species corresponding to bioactive adrenomedullin, but clearly produced other two more intense bands of higher molecular weight (17 and 22 kDa, respectively) in the three cell lines examined. The 22 kDa band was stronger in the αT3-1 extracts than in those of the other two cell lines. When antibodies against PAMP were used in this type of analysis, no band for processed PAMP was observed in any of the three cell lines, although in all of them the two bands for the precursor (17 and 22 kDa) were readily detected by this antibody (Fig. 3A). Northern blot analysis in these cell lines revealed an important quantitative difference in the expression of the 1.6 kb mRNA species among the three cell lines, AtT20 being the one that expresses highest levels, and GH3 the one that shows less abundant adrenomedullin messenger mRNA (Fig. 3B). Strong signal by adrenomedullin-like and PAMP-like immunoreactive material was observed in the three cell lines. No obvious difference in the immunohistochemical intensity among the three was found (Fig. 4A–F).

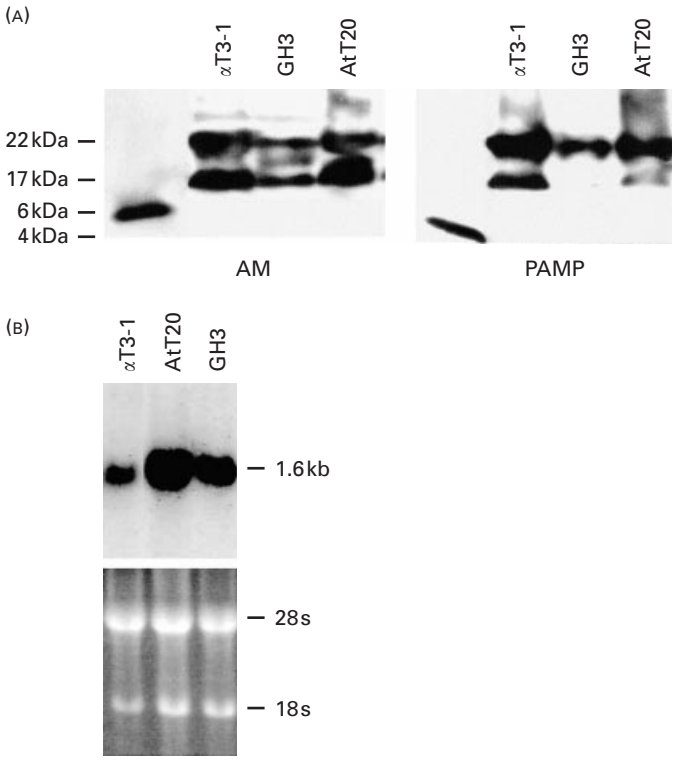

FIG. 3. (A) Western blot analysis of protein extracts of three mouse pituitary cell lines using antibodies against adrenomedullin (AM) and proadrenomedullin N-20 terminal peptide (PAMP). (B) Northern blot analysis of the total RNA of the same three cell lines using a probe specific for mouse AM mRNA. The lower panel shows ethidium bromide staining of the gel before transference to show loading.

Serial sections and ultrastructural studies

In order to further characterize this heterogeneous population of cells, and especially to ascertain which cell type corresponds to the PAMP strongly immunoreactive cells, we conducted immunostaining experiments in serial consecutive paraffin sections with antibodies directed against PAMP and the classical pituitary hormones (data not shown). This approach was useful to discard GH-, PRL-, and ACTH-producing cells as the main PAMP-immunoreactive cell type via the comparison of staining patterns. However, the thickness of the sections precluded cell-to-cell accurate comparison that could show colocalization of both PAMP and any of the classical hormones in the same cell. In order to solve this problem, two technical approaches were carried out: serial semithin sections of plastic embedded material and ultrastructural studies.

In order to find out which of the classical pituitary cell types is expressing higher levels of PAMP in the pituitary,

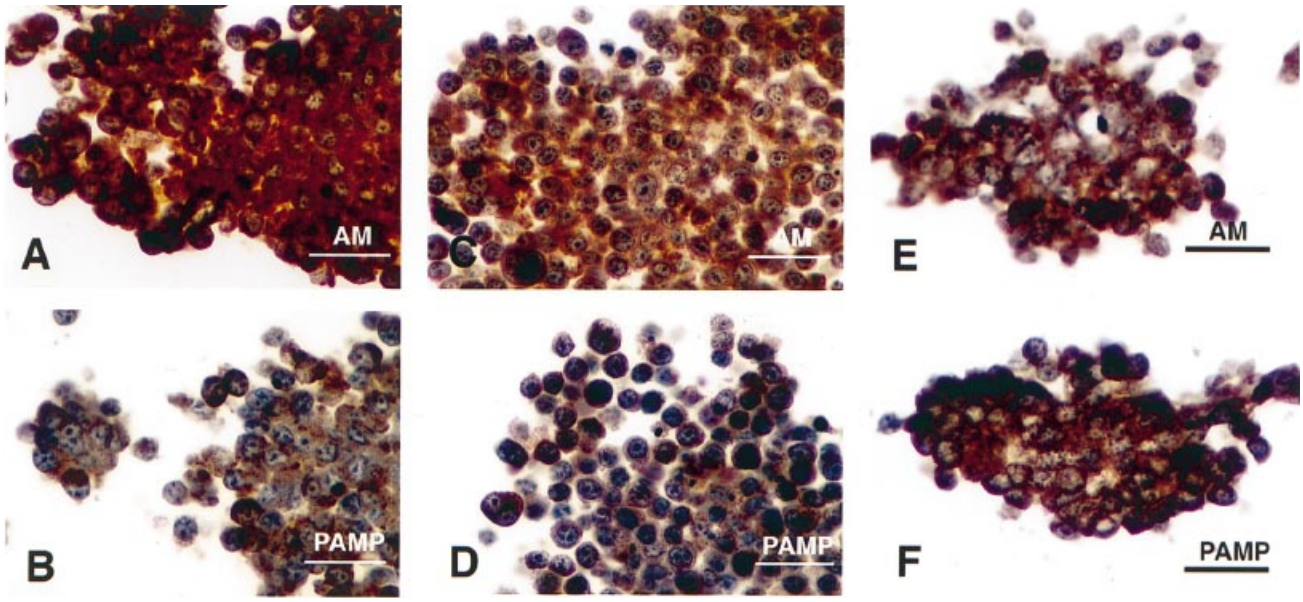

FIG. 4. Immunostaining of sections of pellets of the same three cell lines as Fig. 3. (A, B) AtT20; (C, D) GH3; (E, F) αT3-1 using antibodies against adrenomedullin (AM) (A, C, E) and proadrenomedullin N-20 terminal peptide (PAMP) (B, D, F) × 720 bar = 20 μm.

we studied semithin and thin sections. Although some degree of antigenicity loss is usually caused by the plastic embedding procedures, we trusted that the high levels of staining observed at the paraffin level would permit visualization of the PAMP immunoreactive material in semithin sections and electron microscopy, at least in the subpopulation of strongly immunoreactive cells. Using this approach, we were able to show a clear colocalization of PAMP with FSH–LH containing gonadotropes (Fig. 5). In semithin sections, both very intense and intermediate PAMP immunoreactive cells were observed and serial sections showed also that they were not immunoreactive for PRL, GH, ACTH or TSH (Fig. 5A–F) but colocalized with FSH and, in some cells, with LH (Fig. 5G–J). All the PAMP-immunoreactive cells are also immunostained for FSH and LH. LH cells not immunoreactive for FSH and PAMP were also found with this approach (Fig. 5G,H), together with LH and FSH immunoreactive cells that are negative for PAMP (Fig. 5G–J, double arrow). Crossed absorption controls were carried out by incubation of the anti-PAMP antibodies with either LH or FSH (20 nmol/ml) before the immunostaining procedure. Neither of the two hormones blocked or reduced the immunostaining levels for PAMP. A further confirmation of our results came form the analysis of the localization of PAMP immunostaining at the ultrastructural level (Fig. 6). Colloidal gold immunocytochemistry was carried out in order to confirm the gonadotropic-specific ultrastructural features of the PAMP-immunoreactive cells. This EM approach was used to ascertain which of the two subpopulation of granules present in gonadotropes, known to contain either LH or FSH, expressed the stored PAMP-like material. Our results clearly demonstrated that the only subpopulation of cells in the anterior pituitary which expressed PAMP immunoreactivity at the semithin and ultrastructural level were the gonadotropes. Moreover, immunolocalization at the ultrastructural level showed that the PAMP-immunoreactive material was restricted to the larger FSH-containing granules of the gonadotropes (Fig. 6). Unfortunately, our antibodies against adrenomedullin did not work on semithin plastic-embedded sections or at the ultrastructural level with the colloidal-gold technique in pituitary. Therefore, conclusions about the expression and distribution of adrenomedullin have to be drawn only from the data obtained with the paraffin embedded tissues and cell lines.

Effect of adrenomedullin and PAMP on cAMP in pituitary cell lines

Adrenomedullin and PAMP are secreted peptides which may have either local (autocrine–paracrine) or distant (endocrine) actions. In order to get an idea of the possible target of the secreted peptide, we have performed experiments of cAMP stimulation of the mouse pituitary cell lines with adrenomedullin or PAMP. Both adrenomedullin and PAMP failed to stimulate cyclic AMP production in the three cells lines that were readily stimulated by either PACAP (Fig. 7) or forskolin (data not shown).

Discussion

In the present paper, we have shown the expression of adrenomedullin and its related peptide PAMP in the mammalian pituitary. We have also demonstrated a very noticeable expression of PAMP-immunoreactive material in the gonadotropes which is consistent with previously reported radioimmunoassay data on PAMP in the pituitary (9). Prior studies on mammalian tissue extracts have identified a significantly higher PAMP/adrenomedullin ratio in the pituitary as compared to other organs in which both peptides have been found (9). Our report is the first to show PAMP

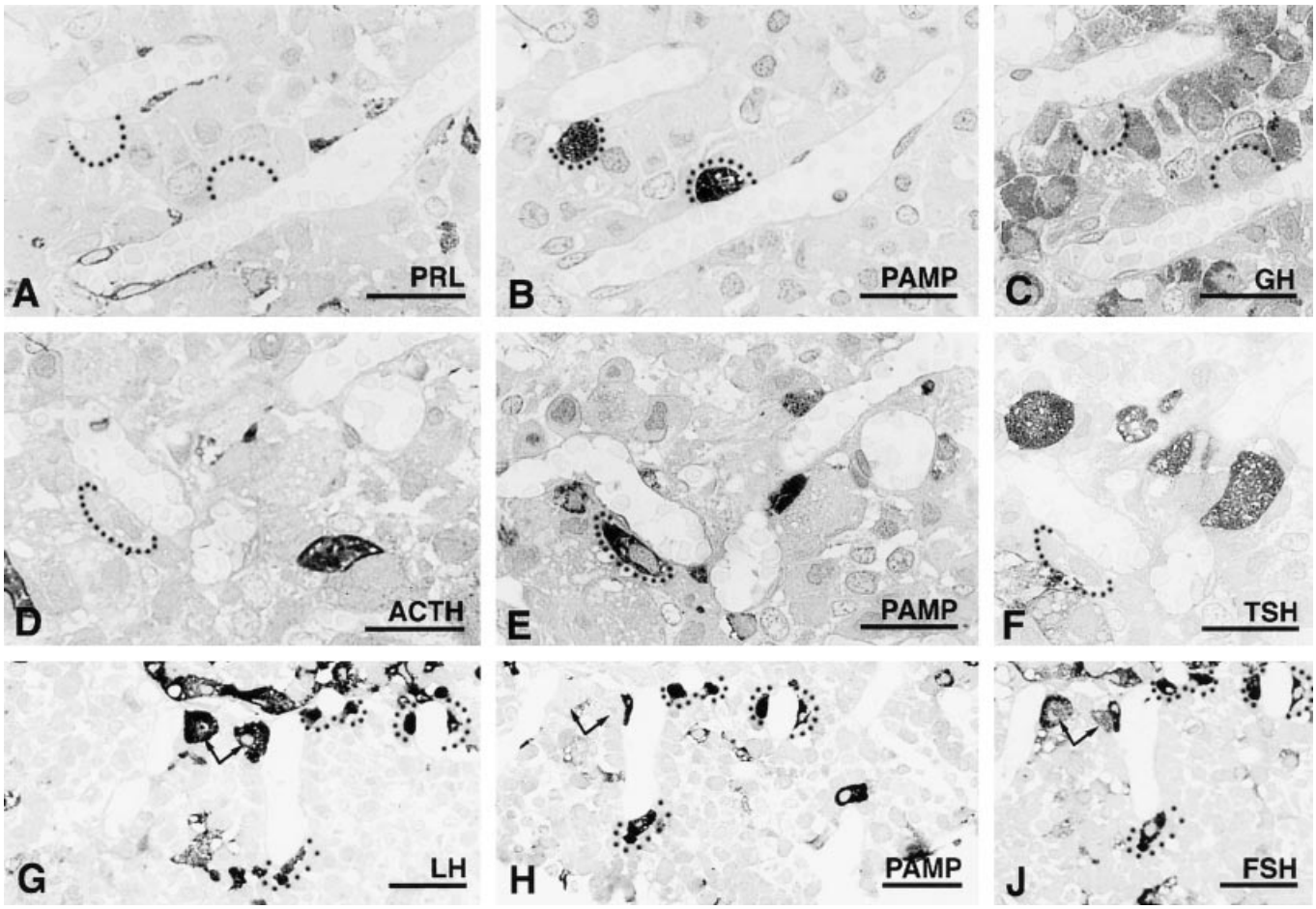

FIG. 5. Three series of semithin serial sections of rat anterior pituitary (A–C, D–F, G–J) immunostained for PAMP (central panels B, E and H) and other pituitary hormones, namely prolactin (PRL) (A), growth hormone (GH) (C), adrenocorticotropic hormone (ACTH) (D), thyroid stimulating hormone (TSH) (F), luteinizing hormone (LH) (G) and follicle stimulating hormone (FSH) (J). Some of the proadrenomedullin N-20 terminal peptide (PAMP) immunoreactive cells, present in the three sections of each row, are marked by a dotted line. Note that the PAMP immunoreactive cells are not immunoreactive for PRL, GH, ACTH or TSH but are simultaneously revealed by antibodies against FSH and LH. Some LH/FSH cells are negative for PAMP (arrows in angled doublet G–H). (A–F) $\times 1800$ bar = 10 μm; (G–J) $\times 720$ bar = 20 μm.

immunoreactivity in pituitary and its localization in FSH/LH cells and the first to demonstrate by western blot that adrenomedullin precursor is expresed and processed in human pituitary. All of our western analysis show two bands at 22 kDa and 17 kDa. The 22 kDa product may correspond to the preproadrenomedullin peptide (185 amino acids) and the 17 kDa band could correspond to an intermediate product of a first cleavage but still a precursor molecule that may contain the amino acid sequences of both adrenomedullin and PAMP peptides, as is shown by the immunoreaction with both antibodies. In any case, none of these precursor peptides have been isolated and sequenced yet. We intend to do so in the near future.

Our immunocytochemical results using anti-adrenomedullin antibodies are in line with our previous reports on foetal rat and mouse embryogenesis (24) and with a recent report on general distribution of adrenomedullin in rodents using *in situ* hybridization techniques (29). Among many other organs, these reports show expression of adrenomedullin both in the anterior and posterior lobes of the pituitary. On the contrary, the present data is in partial conflict with prior observations by Washimine *et al.* (19). In their 1995 report, an overview of immunocytochemical analysis of adrenomedullin in a variety of human, rat and pig organs, including anterior and posterior pituitary, under their experimental conditions, no immunoreactivity was found for adrenomedullin in the posterior pituitary of any of the three species studied, nor in the anterior pituitary of the rat. In the anterior lobe of human and porcine pituitary, the authors found adrenomedullin immunoreactive cells with a distribution different from GH, PRL and ACTH cells. Our results do not exclude that cells belonging to these cell types express adrenomedullin. Although so far we have not been able to achieve good immunoreactivity for semithin plastic embedded sections with our anti-adrenomedullin antibody, we aim to try new strategies to find out the correlation between adrenomedullin-immunoreactivity and the classical pituitary hormones. The differences found between our immunocytochemical results and those of Washimine *et al.*, namely their lack of immunoreaction in rat pituitary compared to the abundant expression shown in our data, may be due to the different characteristics of the antibodies used. In fact, these

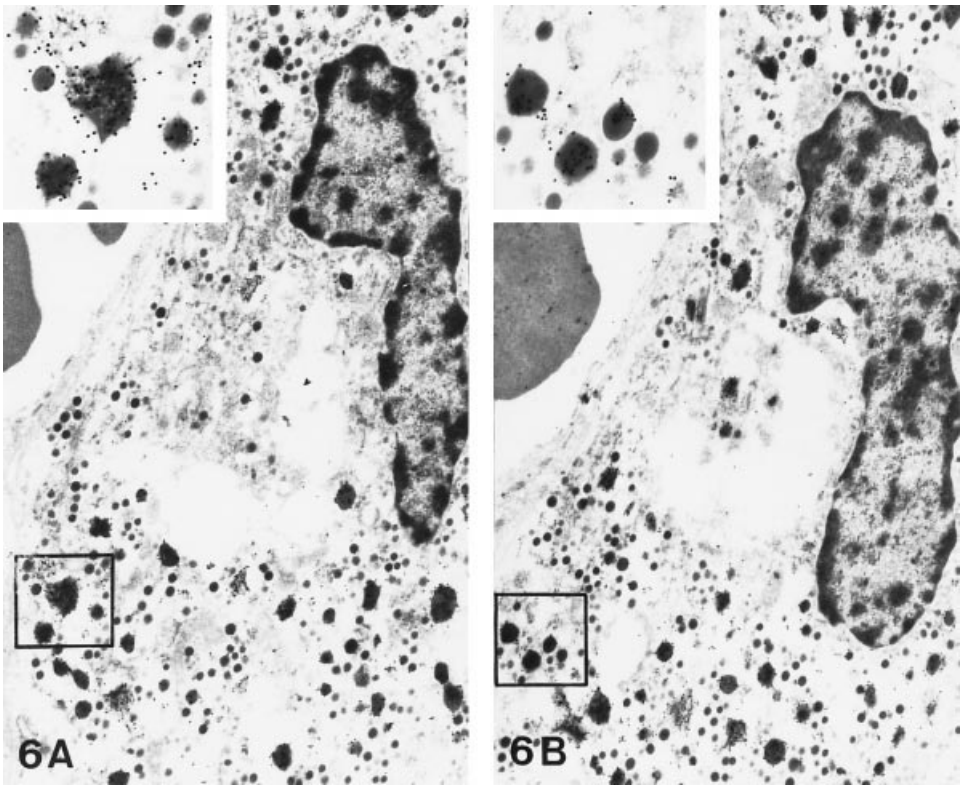

FIG. 6. Serial thin sections of rat pituitary immunostained for proadrenomedullin N-20 terminal peptide (PAMP) (A) and follicle stimulating hormone (FSH) (B). The same cell is immunoreactive for both antibodies. Note that the insets are enlargements of the squared areas in (A) and (B). The colloidal gold particles are clearly seen in the larger granules of the cell. (A, B × 13,000, insets × 30,000).

authors also report negative staining in organs such as brain, kidney or heart, which have now been shown, by other laboratories using different antibodies, to contain immunoreactive adrenomedullin (13, 30, 31).

The lack of complete overlapping of adrenomedullin and PAMP immunoreactivity that we have found, may be due to cell-specific processing of the preproadrenomedullin molecule in each pituitary cell type, in particular the gonadotropes. All the data available to date on the expression of PAMP in mammals show that the concentration of this peptide in the plasma or in tissues is always lower than that of adrenomedullin. For example, the average human plasma adrenomedullin concentration in normal adults is around 5.5 ± 0.21 fmol/ml (15), whereas PAMP is 0.78 ± 0.12 fmol/ml (8). Consistent with these data, in our hands immunocytochemical staining using antibodies against PAMP in other organs always render much fainter signal compared to the results using antibodies against adrenomedullin (e.g. 24). The pituitary gonadotropes are a very striking exception to this rule, with a more intense signal for PAMP than for adrenomedullin, suggesting that an important difference exist in the processing of the adrenomedullin precursor protein in this cell type. Cell type-specific differences in the processing of preproadrenomedullin have also been suggested to explain the significant decrease of the PAMP/adrenomedullin ratio in protein extracts of pheocromocytoma (20%) when compared to normal human adrenals (39%) (32). Our own unpublished results also show a marked variability in the relative abundance of the different electrophoretically separable adrenomedullin-and PAMP-immunoreactive species in cow pituitary and adrenals, also suggesting organ-specific postranslational regulation. An adenohypophysis-specific processing of preprocholecystokinin, with most of the propeptide processed to immature non amidated large peptides, instead of the small amidated products that predominate in the principal production sites of gastrin and cholecystokinin-8, has also been reported (33). Although cell-specific post-translational regulation of peptides is a well-established phenomenon, for example in the proopiomelanocortin derived peptides (e.g. 34), very little data are available about this fact in relation to the proadrenomedullin derived peptides. One of the enzymes required for postranslational modification of adrenomedullin precursor, peptidyl-glycine α-amidating monooxygenase (PAM), is also widely expressed in the anterior pituitary and preferentially produced by the gonadotropes (35).

The gonadotropes are particularly rich in a PAMP-immunoreactive species and there is a significantly higher expression of this molecule in the subtype of gonadotropes that accumulate FSH, than in those that are mainly LH secretors. Heterogeneity within a given cell type in the pituitary cell population is a well established phenomenon that occurs mainly in the somatotrope/lactotrope and in the gonadotrope cell populations (36). The possible cosecretion of this PAMP-immunoreactive material and LH/FSH was further supported when we performed immunocytochemical techniques at the ultrastructural level, showing the very precise localization of

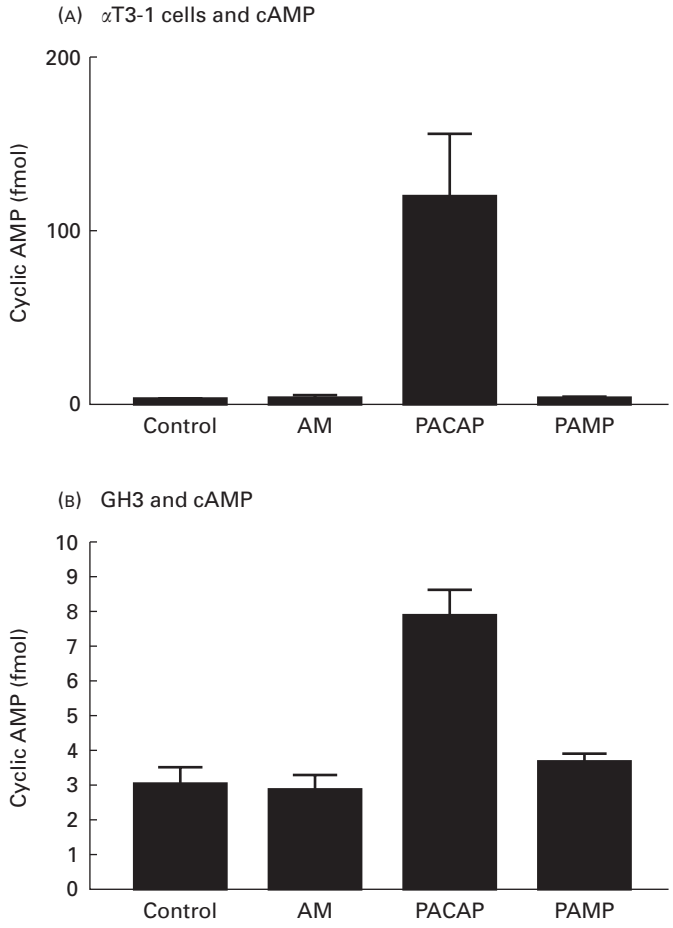

FIG. 7. Effect of adrenomedullin (AM) and proadrenomedullin N-20 terminal peptide (PAMP) on cAMP production in mouse pituitary cell lines. (A) Effect on αT3-1. (B) Effect on GH3. Effect on AtT20 is not shown but was also non significant for both AM and PAMP.

the PAMP-immunoreactive material to the gonadotrope secretory granules, especially those of larger size. Furthermore, the sex-specific differences in the expression of PAMP are parallel to what has already been described for FSH-containing cells in rat pituitary. A thorough light microscopy and ultrastructural characterization of the heterogeneous rat gonadotrope population has recently been published (37) showing two main types of morphologically and immunoreactivelly distinct granules: a small round electrondense granule with an average diameter of 200 nm and a larger less opaque granule of 400–700 nm, the former type being only immunoreactive to LH antibodies and the later accumulating both LH and FSH. PAMP immunoreactivity was mainly localized to the larger FSH containing granules, suggesting a possible simultaneous release of PAMP and FSH upon stimulation, and also supporting a possible involvement of PAMP or adrenomedullin in the regulation of reproductive functions. Changes in the expression of adrenomedullin in the female reproductive organs, in parallel with the reproductive cycle have already been reported (38). The high concentration of PAMP in gonadotropes does not exclude these and the other cell types making adrenomedullin. In fact, immunostaining for adrenomedullin is more widespread than that of PAMP, although not so intense. Consistent with this widespread expression of adrenomedullin in the several cell types that comprise the pituitary, adrenomedullin mRNA and both adrenomedullin and PAMP protein were shown to be abundant in the three cell lines studied GH3, AtT20 and αT3-1 that are derived, respectively, from mouse somatotropes/lactotropes, corticotropes and gonadotropes.

A detailed analysis of the promoter region of the human and mouse adrenomedullin genes (Genbank accession nos. D43639 and D78349, respectively) suggests that a number of putative binding sites for well established pituitary-specific transcription factors are present, giving further support to our finding of high expression of this gene in pituitary tissues and cell lines. Both in human and mouse adrenomedullin gene promoter, at least one potential gonadotropic-specific element (GSE), a binding site for steroidogenic factor-1 (SF-1), was found at −779, −754 (reverse) in human and −729 in mouse (Fig. 8). SF-1 is a member of the steroid receptor superfamily that has been shown necessary for gonadotrope differentiation within the pituitary. SF-1 is a transcriptional activator of the human α-subunit, the gonadotropin-releasing hormone receptor and the LH-β genes

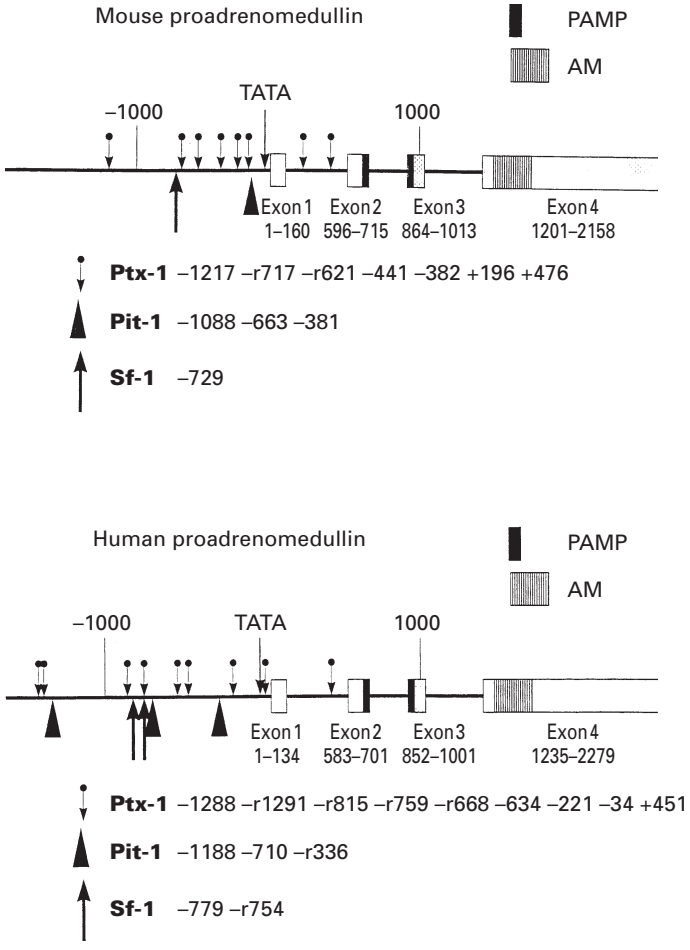

FIG. 8. Potential binding sites for pituitary-specific transcription factors in the mouse and human preproadrenomedullin gene.

(39–41) in the gonadotropes and is highly expressed in this cell type (42) but not in the other pituitary endocrine cells. These findings and the localization of a proadrenomedullin-derived peptide found in our study in the gonadotropes suggest that SF-1 may be implicated in the regulation of adrenomedullin-gene expression through the GSE present in its promoter. Putative consensus sequences for other adenohypophysis-specific transcription factors have also been found in the 5′ adrenomedullin promoter region of both human and mouse genes [for a review on pituitary transcription factors, see (43)]. Pit-1, a helix-turn-helix homeobox protein has been the object of numerous studies and has been shown to regulate the expression of the GH, PRL, TSHα and growth hormone-releasing hormone genes. One Pit-1 putative binding sequence was already reported in the mouse adrenomedullin gene promoter position (−381) (6). In the same promoter region, several sequences that resemble the Pit-1 consensus binding site with only one base mismatch, can be located (Fig. 8). In the human promoter region several putative Pit-1 binding sequences with only one mismatch have also been found. In both human and mouse adrenomedullin-gene promoter, a variety of putative pituitary homeobox 1 (Ptx-1) pan-pituitary activator transcription factors are found in the proximity of both the SF-1 and the Pit-1 putative binding sequences. Ptx-1 has been shown to activate most of the pituitary hormone gene promoters, and is abundantly expressed in several pituitary cell types and cell lines including αT3-1 and AtT20 (44). Interestingly, recent studies have shown a strong sinergistic effect of Ptx-1 and both Pit-1 and SF-1 transcription activators. Further studies, using gel-shift or super-shift approach, should be able to resolve whether these putative binding sequences are important in the selective regulation of adrenomedullin gene expression in the pituitary.

Previous *in vivo* studies have shown inhibition of ACTH release in sheep by adrenomedullin (21). Adrenomedullin suppressed basal and CRF-induced ACTH release from preparations of dispersed rat pituitary cells (20). Recently, an inhibitory effect of PAMP on basal, but not on stimulated ACTH secretion has been shown in the same *in vitro* model (45). Interestingly, the effect seems to mediated by a signal transduction mechanism different to that of adrenomedullin. Our studies on mouse pituitary adenoma cell lines, also failed to shown any stimulation of cAMP by either adrenomedullin or PAMP in any of the cell lines tested, including the corticotrope-derived AtT20. Our data thus do not support the hypothesis of any paracrine effect of adrenomedullin or PAMP in the neighbouring cells through cAMP regulation. The failure of adrenomedullin to stimulate cAMP accumulation in cultured cells is consistent with the initial report of adrenomedullin's action in pituitary cell dispersions (20). Nevertheless, recent reports suggest that autocrine/paracrine influence may exist, at least for PAMP, via alternative signal transduction pathways (calcium influx or phosphatidyl inositol turnover). The fact that both adrenomedullin and PAMP are expressed at high levels in corticotrope-derived AtT20 cells suggest that the reported effects of both peptides on ACTH release could be of an autocrine nature. On the other hand, the colocalization of PAMP in the same granules as FSH appears to support some type of endocrine regulation of a distant target reached via the bloodstream, and maybe an involvement of adrenomedullin-related peptides in the hormonal control of reproductive functions.

In summary, our collective data demonstrate the expression of proadrenomedullin derived peptides in the endocrine cells of the pituitary in a variety of mammalian species. In addition, these peptides have been found in adenohypophysis-derived cell lines. We have also localized PAMP-like immunoreactive material to the FSH-containing granules of the gonadotropes. Our morphological and functional data suggest a possible involvement of the proadrenomedullin derived peptides in the central endocrine control of reproduction.

Acknowledgements

We are very grateful to Drs Betty Eipper and Pammela Mellon for supplying their cell lines; to S. Kahl for his help in sample collection; to the National Hormone and Pituitary Program (NIDDK) for the antibodies and peptides against pituitary hormones. This work was supported in part by the University of Navarra and the Fundación Roviralta.

Accepted 18 October 1999

References

1 Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993; **192:** 553–560.

2 Ishimitsu T, Kojima M, Kangawa K, Hino J, Matsuoka H, Kitamura K, Eto T, Matsuo H. Genomic structure of human adrenomedullin gene. *Biochem Biophys Res Commun* 1994; **203:** 631–639.

3 Wang X, Yue TL, Barone FC, White RF, Clark RK, Willette RN, Sulpizio AC, Aiyar NV, Ruffolo RR Jr, Feuerstein GZ. Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. *Proc Natl Acad Sci USA* 1995; **92:** 11480–11484.

4 Barker S, Wood E, Clark AJ, Corder R. Cloning of bovine preproadrenomedullin and inhibition of its basal expression in vascular endothelial cells by staurosporine. *Life Sci* 1998; **62:** 1407–1415.

5 Kitamura K, Kangawa K, Kojima M, Ichiki Y, Matsuo H, Eto T. Complete amino acid sequence of porcine adrenomedullin and cloning of cDNA encoding its precursor. *FEBS Lett* 1994; **338:** 306–310.

6 Okazaki T, Ogawa Y, Tamura N, Mori K, Isse N, Aoki T, Rochelle JM, Taketo MM, Seldin MF, Nakao K. Genomic organization, expression, and chromosomal mapping of the mouse adrenomedullin gene. *Genomics* 1996; **37:** 395–399.

7 Kitamura K, Sakata J, Kangawa K, Kojima M, Matsuo H, Eto T. Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. *Biochem Biophys Res Commun* 1993; **194:** 720–725.

8 Washimine H, Kitamura K, Ichiki Y, Yamamoto Y, Kangawa K, Matsuo H, Eto T. Immunoreactive proadrenomedullin N-terminal 20 peptide in human tissue, plasma and urine. *Biochem Biophys Res Commun* 1994; **202:** 1081–1087.

9 Insatsu H, Sakata J, Shimokubo T, Kitani M, Nishizono M, Washimine H, Kitamura K, Kangawa K, Matsuo H, Eto T. Distribution and characterization of rat immunoreactive proadrenomedullin N-terminal 20 peptide (PAMP) and the augmented cardiac PAMP in spontaneously hypertensive rat. *Biochem Mol Biol Int* 1996; **38:** 365–372.

10 Kanazawa H, Kurihara N, Hirata K, Kudoh S, Kawaguchi T, Takeda T. Adrenomedullin, a newly discovered hypotensive peptide, is a potent bronchodilator. *Biochem Biophys Res Commun* 1994; **205:** 251–254.

11 Yamaguchi T, Baba K, Doi Y, Yano K. Effect of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. *Life Sci* 1995; **56:** 379–387.

12 Martínez A, Weaver C, López J, Bhathena SJ, Elsasser TH, Miller MJ, Moody TW, Unsworth EJ, Cuttitta F. Regulation of insulin secretion and blood glucose metabolism by adrenomedullin. *Endocrinology* 1996; **137:** 2626–2632.

13 Jougasaki M, Wei CM, Aarhus LL, Heublein DM, Sandberg SM, Burnett JC. Renal localization and actions of adrenomedullin – a natriuretic peptide. *Am J Physiol* 1995; **268:** F657–F663.
14 Miller MJ, Martínez A, Montuenga LM, Garayoa M, Moody T, Unsworth E, Cuttitta F. Adrenomedullin: a pluripotent peptide with growth regulatory function. In: Martínez A, Cuttitta F, eds. *Adrenomedullin.* Amsterdam: IOS Press, 1998: 171–184.
15 Sakata J, Shimokubo T, Kitamura K, Nishizono M, Iehiki Y, Kangawa K, Matsuo H, Eto Y. Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. *FEBS Lett* 1994; **352:** 105–108.
16 Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, Eto T. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett* 1994; **338:** 6–10.
17 Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, Eto T. Distribution and characterization of immunoreactive adrenomedullin in porcine tissue, and isolation of adrenomedullin[26–52] and adrenomedullin[34–52] from procine duodenum. *J Biochem* 1995; **118:** 765–770.
18 Takahashi K, Satoh F, Sone M, Murakami O, Sasano H, Mouri T, Shibahara. Expression of adrenomedullin mRNA in the human brain and pituitary. *Peptides* 1997; **18:** 1051–1053.
19 Washimine H, Asada Y, Kitamura K, Ichiki Y, Hara S, Yamamoto Y, Kangawa K, Sumiyoshi A, Eto T. Immunohistochemical identification of adrenomedullin in human, rat, and porcine tissue. *Histochem Cell Biol* 1995; **103:** 251–254.
20 Samson WK, Murphy T, Schell DA. A novel vasoactive peptide, adrenomedullin, inhibits pituitary adrenocorticotropin release. *Endocrinology* 1995; **136:** 2349–2352.
21 Parkes DG, May CN. ACTH-suppressive and vasodilator actions of adrenomedullin in conscious sheep. *J Neuroendocrinol* 1995; **7:** 923–929.
22 Nussdorfer GG, Rossi GP, Mazzocchi G. Role of adrenomedullin and related peptides in the regulation of the hypothalamo-pituitary-adrenal axis. *Peptides* 1997; **18:** 1079–1089.
23 Luft JH. Improvement in epoxy embedding methods. *J Biophys Biochem Cytol* 1961; **9:** 409–417.
24 Montuenga LM, Martínez A, Miller MJ, Unsworth EJ, Cuttitta F. Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology* 1997; **138:** 440–451.
25 Martínez A, Miller MJ, Unsworth EJ, Siegfried JM, Cuttitta F. Expression of adrenomedullin in normal human lung and in primary tumors. *Endocrinology* 1995; **136:** 4099–4105.
26 Lane BP, Europa DL. Differential staining of ultrathin sections of epon embedded tissues for light microscopy. *J Histochem Cytochem* 1965; **13:** 579–582.
27 Shu S, Ju G, Fan L. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988; **85:** 169–171.
28 Merighi A, Polak JH, Fumagalli G, Theodosis DT. Ultrastructural localization of neuropeptides GABA in rat dorsal horn: a comparison of different immunogold labelling techniques. *J Histochem Cytochem* 1989; **37:** 529–540.
29 Cameron VA, Fleming AM. Novel sites of adrenomedullin gene expression in mouse and rat tissues. *Endocrinology* 1998; **139:** 2253–2264.
30 Ueta Y, Kitamura K, Isse T, Shibuya I, Kabashima N, Yamamoto S, Kangawa K, Matsuo H, Eto T, Yamashita H. Adrenomedullin immunoreactive neurons in the paraventricular and supraoptic nuclei of the rat. *Neurosci Lett* 1995; **202:** 37–40.
31 Jougasaki M, Wei C-M, Heublein DM, Sanderg SM, Burnett JC. Immunohistochemical localization of adrenomedullin in canine heart and aorta. *Peptides* 1995; **16:** 773–775.
32 Kuwasako K, Kitamura K, Ichiki Y, Kato J, Kangawa K, Matsuo H, Eto T. Human proadrenomedullin N-terminal 20 peptide in pheochromocytoma and normal adrenal medulla. *Biochem Biophys Res Commun* 1995; **211:** 694–699.
33 Rehfeld JF. Preprocholecystokinin processing in the normal human anterior pituitary. *Proc Nat Acad Sci USA* 1987; **84:** 3019–3023.
34 Mains RE, Eipper BA. Postranslational procession of neuropeptide precursors. *NIDA Res Monogr* 1984; **54:** 168–183.
35 Steel JH, Martinez A, Springall DR, Treston AM, Cuttitta F, Polak JM. Peptidyl glycine alfa amidating monooxygenase (PAM) immunoreactivity and messenger RNA in human pituitary and increased expression in pituitary tumors. *Cell Tissue Res* 1994; **276:** 197–207.
36 Childs GV. Division of labor among gonadotropes. *Vitam Horm* 1995; **50:** 215–286.
37 Jeziorowski T, Watanabe T, Bargsten G, Grube D. Heterogeneity of pituitary gonadotrope cells in male rats. *Arch Histol Cytol* 1997H; **60:** 355–370.
38 Macri C, Loup D, McHale M, Jacobs R, Bales L, Sundborg M, Amstrong A, Gehlbach D, Mitchell A, Nelson M, Macri C, Miller MJ, Martínez A, Cuttitta A, Gray K. Adrenomedullin is widely expressed throghout normal and abnormal reproductive tissue of woman: evidence of cyclic regulation. In: Martínez A, Cuttitta F, eds. *Adrenomedullin.* Amsterdam: IOS Press, 1998: 207–248.
39 Barnkhart KM, Mellon PL. The orphan nuclear receptor steroidogenic factor-1 regulates the glycoprotein hormone α-subunit gene in pituitary gonadotropes. *Mol Endocrinol* 1994; **8:** 878–885.
40 Duval DL, Nelson SE, Clay CM. A binding site for steroidogenic factor-1 is part of the complex enhancer that mediates expression of the murine gonadotropin-releasing hormone receptor gene. *Biol Reprod* 1997; **56:** 160–168.
41 Halvroson LM, Kaiser U, Chin W. Stimulation of luteinizing hormone β gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. *J Biol Chem* 1996; **271:** 6645–6650.
42 Asa SL, Bamberger A, Cao B, Wong M, Parker KL, Ezzat S. The transcription activator steroidogenic factor-1 is preferentially expressed in the human pituitary gonadotroph. *J Clin Endocrinol Metab* 1996; **81:** 2165–2170.
43 Lloyd RV, Osamura RY. Transcription factors in normal and neoplastic pituitary tissues. *Microsc Res Techn* 1997; **39:** 168–181.
44 Tremblay JJ, Lanctot C, Drouin J. The pan-pituitary activator of transcription, Ptx-1 (pituitary homeobox-1) acts in synergy with SF-1 and Pit-1 and is an upstream regulator of the lim-homeodomain gene Lim3/Lhx3. *Mol Endocrinol* 1998; **12:** 428–441.
45 Samson WK, Murphy TC, Resch ZT. Proadrenomedullin N-terminal 20 peptide inhibits adrenocorticotropin secretion from cultured pituitary cells, possibly via activation of a potassium channel. *Endocrine* 1998; **9:** 269–272.