Distribution of adrenomedullin and proadrenomedullin N-terminal 20 peptide immunoreactivity in the pituitary gland of the frog *Rana perezi*

M. Collantes, M.E. Bodegas,* M.P. Sesma, and A.C. Villaro

Department of Histology and Pathology, University of Navarra, 31080 Pamplona, Spain

Accepted 25 March 2003

Abstract

Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are two multifunctional peptides processed from a common precursor which have been described in numerous mammalian organs, including the pituitary gland. Previous studies have found AM immunoreactivity in neurohypophysis nerve fibers of amphibian pituitary. In the present study, immunocytochemical and Western blot analysis in the pituitary gland of the amphibian *Rana perezi* demonstrated in the adenohypophysis both AM and PAMP. AM-like immunoreactivity was found in a moderate number of endocrine cells of the pars distalis. In the neurohypophysis, AM was observed not only in nerve fibers of pars nervosa and axonal projections innervating the pars intermedia, but also in the outer zone of the median eminence. PAMP staining was observed in numerous endocrine cells scattered all over the pars distalis and in some cells of the pars tuberalis, but not in the neurohypophysis. In order to compare the quantity of AM and PAMP immunoreactivity between pars distalis of female and male specimens, an image analysis study was done. Significant differences for AM immunoreactivity \( (p < 0.001) \) between sexes was found, the males showing higher immunostained area percentage. Differences of PAMP immunoreactivity were not significant \( (p = 0.599) \). Western blot analysis detected bands presumably corresponding to precursor and/or intermediate species in the propeptide processing.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Adrenomedullin; Proadrenomedullin N-terminal 20 peptide; Pituitary gland; Frog; Immunocytochemistry; Western blot

1. Introduction

Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are two regulatory peptides generated by consecutive enzymatic cleavage and amidation from a common precursor of about 185 amino-acids (aa) called preproAM (Kitamura et al., 1993a).

Adrenomedullin is a 52 aa amidate peptide structurally related to the calcitonin gene-related peptide superfamily, which also includes calcitonin, calcitonin gene-related peptide (CGRP) and amylin (Wimalawansa, 1997). AM was isolated from a human pheochromocytoma as a very potent hypotensive molecule (Kitamura et al., 1993b). Since then, AM was found using a larger variety of techniques in different mammalian organs such as adrenal gland, heart, lung, pancreas, kidney, gastrointestinal and reproductive tracts, central nervous system, and pituitary gland (Hinson et al., 2000). Circulating AM has also been demonstrated in plasma (Sakata et al., 1994). Apart from vasoactive effect, diverse biological functions of AM have been reported: bronchodilation (Kanazawa et al., 1994), inhibition of gastric emptying (Martínez, 1997), antimicrobial actions (Allaker et al., 1999), modulation of cell and tissue growth (Belloni et al., 2001) and regulation of the secretion of several hormones including aldosterone (Yamaguchi et al., 1996), insulin (Martínez, 1996b), and catecholamine (Nussdorfer et al., 1997).

The second bioactive product of proadrenomedullin is the amidated 20 amino-acid peptide PAMP (Kitamura et al., 1994). Studies on PAMP are more limited but, as would be predicted, PAMP seems to be expressed in the same organs as AM (Inatsu et al., 1996; Washimine et al., 1994). However, a separate expression of AM and...
PAMP has been reported in rat pituitary (Montuenga et al., 2000) and other organs such as prostate (Jiménez et al., 1999) and kidney (López et al., 1999b), where the authors suggested a cell specific post-translational process. Another possible explanation for such differential expression is the existence of a process of alternative splicing during the maturation of the mRNA (Martínez, 2001). Physiologically, and in general, PAMP acts in parallel with AM, carrying out the same functions as vasodilator, bronchodilator and regulator of hormonal secretion, but using a different signal transduction pathway (Samson, 1999).

Although almost all the studies of AM and PAMP distribution or actions are focused in mammals, the presence of AM and/or PAMP immunoreactivity have been reported in different organs of non-mammalian species as fishes, amphibians, reptiles, and birds (López et al., 1999a; López et al., 1999b; Muñoz et al., 2001b). In addition, the presence of AM immunoreactivity in the starfish *Marthasterias glacialis* suggests a long phylogenetic history for this peptide (Martínez, 1996a).

With respect to the pituitary gland and in the same way, almost all the studies dealing with the expression of AM and PAMP have been carried out in mammals. ProAM mRNA has been detected by in situ hybridization in the posterior and anterior lobes in rat and in mouse (Cameron and Fleming, 1998; Hwang and Tang, 1999), and Northern blot analyses have also demonstrated AM mRNA in human extracts of whole pituitaries (Takahashi et al., 1997). AM and PAMP peptides were detected either by histological or biochemical techniques in the pituitary of several mammalian species. Immunoreactive AM was detected by radioimmunoassays in protein extracts of human, pig and rat pituitaries (Hwang and Tang, 1999; Ichiki et al., 1995; Takahashi et al., 1997). In the same species, immunocytochemical techniques showed immunoreactivity for AM in the *pars nervosa* and some endocrine cells of the *pars distalis* still unidentified (Montuenga et al., 2000). Within this region of the gland, PAMP immunoreactivity was also present in endocrine cells, identified in rat as gonadotropes (Montuenga et al., 2000). Physiological studies indicated that in the *pars distalis* both peptides regulate the secretion of pituitary hormones. Although there is some controversy, it seems that AM is able to regulate ACTH secretion in cultured rat anterior pituitary cells under different conditions (Mimoto et al., 2001; Samson et al., 1995). Basal ACTH secretion from cultured rat pituitary cells is also inhibited by PAMP (Samson et al., 1998). On the other hand, AM is able to act like a GH secretagogue factor in both normal and tumor mammalian pituitary tissue (Nakamura et al., 1998). In the neurohypophysis, AM immunoreactivity is present in fibers coming from neurons of paraventricular and supraoptic nuclei, where AM co-exist with vasopressin or oxytocin (Ueta et al., 2000).

Contrary to mammals, there are no studies about the presence of AM and PAMP in the pituitary gland of non-mammalian vertebrates. AM immunoreactivity has only been studied in the central nervous system of the anuran *Rana perezi*, where AM immunoreactivity is also referred to nerve fibers of the neurohypophysis and *pars intermedia* (González et al., 1998; Muñoz et al., 2001a; Muñoz et al., 2001b). Concerning PAMP, there are no studies at all in non-mammalian pituitary. All those facts prompted us to investigate the expression of AM in frog adenohypophysis and PAMP in the whole pituitary gland of the amphibian *R. perezi*.

2. Materials and methods

2.1. Tissue samples and processing

Pituitary glands of adult male and female specimens of the anuran *R. perezi* were carefully dissected out. For the immunocytochemical analysis, material was fixed by immersion in Bouin’s liquid for 12 h and washed in 70% ethanol followed by further dehydration in more concentrated alcohols and paraffin embedding. Due to the small size of frog pituitaries, and in order to facilitate the orientation of the sections, the glands were adhered by their ventral surface to 2% low melting agarose blocks before paraffin embedding. Then, the solidified agarose blocks were embedded in paraffin prior to sectioning. Paraffin blocks were serially sectioned at 3–5 μm and placed on slides treated with Vectabond (Vector laboratories; Burlingame, CA). On the other hand, some pituitaries were fixed in 0.1% glutaraldehyde plus 4% paraformaldehyde in saccharose-cacodylate buffer for 2 h at 4°C. The material was washed, dehydrated and then was embedded in glycid ether 100 (Serva) in order to obtain semithin sections of 1 μm.

For Western blot, anterior lobes of pituitaries were frozen in liquid N2.

2.2. Antisera and optimal dilutions

For the immunocytochemical detection, several rabbit anti-AM and anti-PAMP antisera were used.

Three antisera (#2469, #2342, and #2075) directed to the synthetic peptide P072 (AM\textsubscript{22–52} amide) were tested. Best results were obtained using anti-AM (#2075), working at an optimal dilution of 1:300. The characterization of this antiserum was reported previously (Montuenga et al., 1997). The same antiserum also gave the best results in Western blot probes, used in this case at 1:2000.

For PAMP immunodetection, four antisera (#2463, #2336, #2337, and #2338) raised to another synthetic peptide called P070 (PAMP\textsubscript{YY 13–20} amide) were compared. The antiserum rendering best results was anti-
PAMP (#2336), and its optimal dilution for immunocytochemical studies was 1:5000. For Western blot detection, this antiserum was used at 1:2000 dilution. The antiserum #2336 was characterized for binding specificity in the present study using an enzyme-linked immunosorbent assay (ELISA). Four groups of test peptides were used: preproAM derived-peptides, AM related peptides, amidated peptides and pituitary hormones. Complete PAMP and P070 protein were used as positive controls and PBS-TM (phosphate buffered saline with 0.05% Tween 20 and 1% milk) as a negative control. Ninety-six-well polyvinylchloride microtiter plates were coated with PBS-TM to minimize non-specific binding. The test peptides diluted at 2 μg/ml were passively absorbed at 4°C overnight. The antiserum was tested at serial 2-fold dilutions ranging from 1:100 to 1:204,800 and incubated for 2 h at room temperature. Plates were washed exhaustively with PBS-T (PBS with 0.05% Tween 20) before the incubation with peroxidase conjugated anti-rabbit antiserum diluted 1:1000. Plates were washed again several times. Finally, as chromogenic substrate, 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma Chemical; St Louis, MO) was used for the enzymatic reaction of peroxidase. ABTS was dissolved in a citrate buffer (0.01 M; pH 4.0). The absorbance of the final green product was read at 405 nm. Slight cross-reactivity was observed with the amidate peptide IBE2, but this was completely abolished when the antiserum was preabsorbed with the peptide at 1 nmol/ml. The rest of the peptides tested gave negative results, except for PAMP and P070.

2.3. Immunocytochemical technique

For the immunocytochemical localization of AM in paraffin and semithin sections, best results were obtained with EnVision System (Dako K4011). The sections were deparaffinized, rehydrated, and endogenous peroxidase was inhibited in a 3% H2O2 solution in deionized H2O for 10 min. In order to block unspecific background staining, paraffin sections were incubated with normal goat serum (1:20) during 1 h at room temperature before overnight incubation with the specific antiserum at 4°C. Sections were then treated for 30 min with an immunocomplex constituted by a goat anti-rabbit antiserum conjugated with a dextran polymer. After rinsing in Tris-buffered saline (TBS), the peroxidase activity was demonstrated using diaminobenzidine and H2O2 as chromogen and substrate, respectively. This reaction was intensified mixing two solutions: one containing 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), 40 mg of ammonium chloride (Sigma) and 30 mg of glucose oxidase (Sigma), in 50 ml of acetic buffer (acetic acid 0.1 M pH 6). Finally, the sections were counterstained with Harris hematoxylin, dehydrated and mounted with DPX. To improve immunoreaction of AM, a microwave pretreatment was applied before the incubation with the normal serum. Sections were immersed in 0.01 M citric acid (Sigma) buffer, pH 6, and heated for 15 min at 750 W, followed by 15 min at 375 W. Finally, the slides were cooled in running water.

On the other hand, the avidin–biotin complex (ABC) technique was employed for the immunodetection of PAMP. Background of the paraffin sections was blocked using normal pig serum (1:20) for 1 h at room temperature. Then, sections were incubated overnight with the specific antiserum at 4°C. After that, sections were washed in TBS and incubated for 30 min at room temperature with biotinylated pig anti-rabbit IgG (Dako E0355) diluted to 1:200. The sections were then again washed in TBS and incubated for 1 h at room temperature with the ABC (Dako K0355) diluted 1:100. After the final wash in TBS, the immunoreaction was visualized using the same method described above.

Several immunocytochemical controls were done in order to test the specificity of the antisera. Some sections were incubated with TBS or preimmune serum as first layer. On the other hand, specific antisera were preabsorbed overnight at 4°C with an excess of the corresponding antigen (1–10 nmol per each milliliter of diluted antiserum). The sections were incubated with the pre-absorbed antiserum as primary layer. For the pre-absorption controls, the peptides P070 and P072 were used to block the specific binding. All the controls abolished the immunostaining of the tissue.

2.4. Image analysis

In order to compare AM and PAMP immunostaining between pituitaries of female and male specimens, an image analysis study was done. The ratio calculated was the immunostained area fraction (IAF) for AM or PAMP (AM-IAF; PAMP-IAF). The IAF is a ratio between immunostained area and the total area of pars distalis. It was expressed as a percentage, and was calculated as follows. Pituitary glands of ten male and ten female specimens were processed and transversal sections were made. In order to achieve a correct selection of the sections for the quantitative study, a random systematic sampling was done, choosing one out of five sections from each pituitary gland and obtaining about 10 sections per gland. Then, immunocytochemical techniques were applied. Three or 4 light microscopic fields per section were captured by a videocamera connected to a computer. For each field, the reference area (pars distalis) was selected and analyzed by the Visilog v. 4.0 program (Noesis, SA). All the values of immunoreactive and total reference areas, and the ratio between them, were calculated automatically by the software program in each field of the corresponding section. Six
sections per animal were analyzed, and finally, each animal was represented by a mean of all its IAF values. For statistical analysis, significant between group differences \( (p < 0.05) \) were determined by Student’s t test. Data are shown as means and standard errors of the means.

2.5. Western blot

To extract the proteins, the frozen anterior lobes were ground into powder with two frozen metal surfaces. Then, the product was immersed in a buffer containing 151 mM NaCl, 1% deoxicholate, 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1% SDS, 1% Triton X-100 and a cocktail of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Germany). The samples were then centrifuged and the final protein concentration was determined (BCA kit; Pierce, Rockford, IL). Protein extracts were diluted to a concentration of 30\( \mu \)g/20\( \mu \)l, heated to 70°C for 10 min and loaded into a sample well. The samples were separated electrophoretically on a gradient NuPAGE 4–12% bis–tris gel (Invitrogen) and run 200 V for 30 min. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a polyvinylidifluoride membrane (Immobilion PVDF; Millipore, Bedford, MA) at 30 V for 45 min. Membranes were blocked in 1% BSA-PBS for 1 h and then, incubated overnight in 1:2000 dilution of rabbit anti-AM or rabbit anti-PAMP. Exhaustive washes with PBS-T were performed and then a secondary antiserum conjugated with horseradish peroxidase diluted 1:4000 was added during 1 h. Three exhaustive washes were necessary before developing the membranes by an enhanced chemiluminescence detection. The procedure was performed following the instructions described by the manufacturers of the kit (Lumi Light, Roche). Protein extracts from bovine adrenal gland, where AM is expressed in high levels, were used as positive control of AM detection. PAMP analysis was controlled using a synthetic peptide (4291-V, Peptide Institute, INC). Absorption controls using specific antisera preabsorbed with the synthetic P072 and P070 peptides as primary antiserum were done.

3. Results

3.1. Immunocytochemical study

Using specific antisera, AM and PAMP-like immunoreactivity was demonstrated in R. perezi pituitary gland (Figs. 1 and 2).

Immunocytochemical staining for AM was detected both in the adenohypophysis (Figs. 1A–E) and neurohypophysis (Figs. 1F and G). The immunoreactivity was abolished by the absorption controls (Figs. 1B and C).

In the adenohypophysis there was a moderate number of endocrine positive cells in the pars distalis, mainly located in the ventral region (Fig. 1A). The AM positive cells showed variable—strong or moderate—degree of immunoreactivity (Figs. 1D and E). No immunostained endocrine cells were present neither in the pars intermedia nor in the pars tuberalis, while some immunostained fibers were observed in the pars intermedia (Fig. 1F). In the neurohypophysis, immunoreaction was found in nerve fibers of the median eminence and pars nervosa, being absent from the pituicytes (Fig. 1F). In the median eminence, the fibers of the outer zone presented a strong immunoreaction (Fig. 1G).

On the other hand, PAMP immunoreactivity was detected in the adenohypophysis but not in the neurohypophysis (Fig. 2). PAMP staining was found in endocrine cells of the pars distalis (Figs. 2A–E) and pars tuberalis (Figs. 2F and G), but not in the pars intermedia (Figs. 2A, D, and F). In the absorption tests, the immunostaining was completely eliminated. (Figs. 2B and C). In comparison, the number and distribution of PAMP cells in the pars distalis differed from that of the AM cells. PAMP immunoreactive cells were more numerous and more largely distributed all over the pars distalis. PAMP immunoreactive cells with different immunostaining intensity were also observed (Figs. 2D and E).

When AM immunoreactivity was studied in various sections of several specimens, differences in the quantity of AM immunoreactivity were observed, whereas no differences were observed for PAMP (Fig. 3). Then, an image analysis study was done in order to determine if the differences between AM or PAMP was caused by sex. Image analysis with relative measurements of IAF revealed significant differences \( (p < 0.001) \) between sexes in the immunocytochemical detection of AM, the males showing higher IAF than the females (Fig. 4). This result was not observed for PAMP, whose differences of immunostaining were not significant \( (p = 0.599) \) (Fig. 4).

3.2. Western blot analysis

To confirm the immunocytochemical finding of AM and PAMP, Western blot analysis was performed (Fig. 5). Protein extracts from bovine adrenal gland and a synthetic peptide were used as positive controls for AM and PAMP respectively. In the adrenal gland extracts two AM immunoreactive bands of 6 and 13 kDa were found, the 6 kDa band corresponds to the completely processed peptide of 52 aa. The synthetic PAMP of 20 aa originates a 3 kDa band.

When total proteins extracts from Rana perezi pars distalis were studied, three immunoreactive bands for AM in approximately 22, 20, and 15 kDa positions were found (Fig. 5). The 22 and 15 kDa bands always had a very strong immunoreactivity, while the band of 20 kDa was weaker and not always present. On the other hand,
Fig. 1. AM immunoreactivity in the pituitary gland of *R. perezi*. (A) Parasagittal section of the pituitary gland showing distribution of AM staining in pars distalis, pars nervosa, pars intermedia and median eminence. In the pars distalis, most of the positive cells are located in the ventral area. In median eminence, pars nervosa and pars intermedia, immunoreaction was found in nerve fibers, shown at higher magnification in F and G. (B,C) Absorption control in paraffin sections. Immunoreaction for AM (B) is abolished in the serial section using the preabsorbed AM antiserum (C). (D,E) Paraffin (D) and semithin (E) sections of frog pars distalis. Endocrine cells with medium (stars) and high (thin arrows) degrees of staining can be observed. F: In the pars nervosa, immunostained nerve fibers with varicosities (thin arrows) are present. Some immunoreactive fibers enter into the pars intermedia (arrows). (G) In the outer zone of the median eminence (arrows), numerous AM immunostained nerve fibers are observed. PD, pars distalis; PI, pars intermedia; PN, pars nervosa; ME, median eminence. Arrowheads: melanin A, 125×; B, C, and G, 250×; D–F, 500×.
Fig. 2. PAMP immunoreactivity in the pituitary gland of *R. perezi*. (A) Parasagittal section of pituitary gland immunostained for PAMP. Abundant positive cells, scattered all over the *pars distalis*, can be observed. (B,C) Absorption control for PAMP immunoreactivity. Immunostaining for PAMP (B) is not obtained with the preabsorbed PAMP antiserum (C). (D,E) Cells with medium (star) and high (thin arrows) degrees of PAMP immunostaining can be observed, in both paraffin (D) and semithin (E) sections of *pars distalis*. (F) Low magnification micrograph of *pars tuberalis* (arrows) showing positive PAMP cells. (G) Detail of the *pars tuberalis* of another gland containing cells immunostained for PAMP (thin arrows). PD, *pars distalis*; PI, *pars intermedia*; PN, *pars nervosa*; ME, median eminence. Arrowheads: melanin A, 125×; B, C, and F, 250×; D, E, and G: 500×.
Fig. 3. Differences in AM and PAMP immunoreactivity between males and females pituitaries. Representative sections of male (A,C) and female (B,D) pituitaries immunostained for AM (A,B) and PAMP (C,D). Details of pars distalis are shown in the insets. AM cells are more numerous in males (A) than in females (B). On the contrary, no differences in the quantity of PAMP cells in both sexes are observed (C,D). Note PAMP positive cells of the pars distalis are always more numerous than AM cells in both males and females pituitaries. PD, pars distalis; PI, pars intermedia; PN, pars nervosa. A–D, 50×. Insets: 250×.

Fig. 4. Graphic representation of the differences of immunostaining area fraction (IAF) in pituitary gland between males and females for AM (A) and PAMP (B). Significant differences for AM immunoreactivity ($p < 0.001$) between sexes was found, the males showing higher IAF. No significant differences ($p = 0.599$) can be observed for PAMP-IAF.

Fig. 5. Western blot analysis of AM or PAMP in pituitary extracts of R. perezi. For AM study, bovine adrenal gland (AG) was used as a positive control. AG shows 15 and 6 kDa bands, the latter corresponding to the mature bioactive AM. In pituitary extracts of R. perezi (P) AM antiserum detects three bands of 22, 20, and 15 kDa, larger than the 6 kDa one. Such bands presumably correspond to the preproAM precursor and/or intermediates in the propeptide processing. For PAMP study, the synthetic PAMP peptide used as positive control (C+) produced a 3 kDa band. In frog pituitary extracts, a PAMP immunoreactive band of 18 kDa was detected. As in the case of AM, no detectable processed peptide was found.
The presence of AM and PAMP immunoreactivity in frog pituitary is consistent with previous studies in mammals, including human, rat, pig, and cow (Montuenga et al., 2000; Washimine et al., 1995). In all those species, as occurred in *R. perezi*, AM immunoreactivity was found in a sub-population of endocrine cells of the pars distalis, which are still unidentified. On the other hand, PAMP immunoreactivity in frog pituitary was also localised in endocrine cells of the *pars distalis*. In rat, the PAMP immunoreactive cells correspond to gonadotropes, where the peptide was found in the secretory granules which contain follicle stimulating hormone (FSH) (Montuenga et al., 2000). Although further studies will be necessary to find out the physiological actions of AM and PAMP in frog pituitary, the presence of AM and PAMP in the endocrine cells of the *pars distalis* could be related to the regulation of hormone secretion, as occurs in rat pituitary (Samson et al., 1995). The local expression of AM and PAMP in frog pituitary demonstrated one band of approximately 18 kDa. This 18 kDa band could possibly correspond to another intermediate peptide of the post-translational processing of the preproAM to PAMP. In human and mouse pituitary extracts 22 and 17 kDa bands were detected (Montuenga et al., 2000). As in the case of AM, the fully processed 20 aa PAMP peptide of approximately 3 kDa was not detected in the pituitary of any species.

One possibility to explain the lack of mature 6 kDa AM and 3 kDa PAMP is that the final maturation proteolytic steps occur after secretion of intermediates from endocrine cells. A rapid secretion of the mature peptides can also occur. This argument is supported by the fact that Western blot analysis of the mammary gland tissue only shows bands corresponding to the precursors, whereas processed 6 kDa AM is detected in milk (Jahnke et al., 1997). The storage forms of the peptides within the cells, detected by immunocytochemistry in the anterior lobe, could either correspond to the preproAM and/or to processed intermediates. However, the lack of fully processed AM or PAMP Western blot signal does not necessarily imply their absence in frog pituitary. Mature peptides could be present in small quantities, but below their immunodetection threshold. In addition, the existence of larger bioactive forms, still unknown, can also be considered.

Contrary to human and mouse, where common immunoreactive 22 and 17 kDa bands were found in Western blot analysis for AM and PAMP, in frog pituitary no common precursor band was found. Apparently, different precursors for AM and PAMP exist in frog pituitary, therefore suggesting different processing pathways in mammals and frog.

**4.2. AM and PAMP in the adenohypophysis**

The presence of AM and PAMP antiserum detected a band of about 18 kDa. Absorption controls abolished the immunoreaction for AM and PAMP, demonstrating the specificity of the labeling.

**4. Discussion**

The present study of frog pituitary demonstrated for the first time the immunoreactivity of AM and PAMP in the anterior lobe of amphibians using techniques for immunodetection both “in situ” and in protein extracts. To our knowledge, this constitutes the first reference of the two peptides in adenohypophysis, namely in endocrine cells, of non-mammalian vertebrates. The presence of AM immunopositive nerve fibers in the neural and intermediate lobes of amphibians previously reported (González et al., 1998; Muñoz et al., 2001a; Muñoz et al., 2001b) has also been confirmed.

The specificity of such antisera has been demonstrated by previous and present results. AM antiserum used in this study was previously characterized (Montuenga et al., 1997), and PAMP antiserum characterization was performed in our laboratory, showing a negligible cross-reactivity with many other related peptides. In addition, preabsorption of the antiserum with the corresponding synthetic peptides eliminated positive results in both immunocytochemical and Western blot analysis. Despite the fact that the antisera were obtained against human AM or PAMP portions, they appeared to detect well the corresponding peptides of amphibian. In fact, both antisera have been used previously to detect those molecules in other non-mammalian species (López et al., 1999a; López et al., 1999b; Martínez, 1996a) and also to detect AM in the central nervous system of *R. perezi* (Muñoz et al., 2001a; Muñoz et al., 2001b).

**4.1. Molecular forms of AM and PAMP**

The analysis of AM in the frog pituitary showed three bands of about 22, 20, and 15 kDa, but did not detect the processed 6 kDa AM molecule. Previous Western blot analysis of human pituitary and mouse pituitary cell lines also stated the presence of AM-immunoreactive species of 22 and 17 kDa (Montuenga et al., 2000), while only in some human samples slight amounts of the 6 kDa band were demonstrated. Similar high molecular weight bands were detected in Western analysis of other tissues such as lung (Martínez, 1995), kidney (López et al., 1999b), mammary gland (Jahnke et al., 1997), and tumoral cell lines (Miller et al., 1996). Although none of these bands have been sequenced yet, all the authors suggest that these bands correspond to the preproAM peptide or some intermediate products of the post-translational processing of the precursor. On the other hand, the Western blot analysis of PAMP in frog pituitary demonstrated one band of approximately 18 kDa. This 18 kDa band could possibly correspond to another intermediate peptide of the post-translational processing of the preproAM to PAMP. In human and mouse pituitary extracts 22 and 17 kDa bands were detected (Montuenga et al., 2000). As in the case of AM, the fully processed 20 aa PAMP peptide of approximately 3 kDa was not detected in the pituitary of any species.
within the pars distalis provides the possibility of autocrine or paracrine mechanisms of action.

**Differential expression.** Interestingly, the distribution of AM and PAMP immunoreactivity in the pars distalis did not completely overlap, and therefore it is expected that the cell types involved in the production of each peptide were different. In addition, the number of PAMP positive cells is higher than that of AM. These data indicate that, similarly to rat hypophysis (Montuenga et al., 2000), a differential expression of both peptides occurs in frog pituitary. Further research is needed to find out if such differences are due to either alternative splicing (Martínez, 2001) or post-translational processing.

**Males and females.** According to our results, AM immunoreactivity was statistically more abundant in the pars distalis of frog males, whereas the number of PAMP immunoreactive cells was similar in both sexes. In the rat pituitary, no sex related differences of AM immunoreactivity have been detected, while PAMP expression is higher in males (Montuenga et al., 2000). Sexual dimorphism in the immunoreactive concentrations of other regulatory peptides has also been observed in rat pituitary [galanin (Gabriel et al., 1989)/ substance P (Rugarn et al., 1999)]. Similarly, endothelin 3 immunoreactivity has been reported to be preferentially detected in the female pituitary of the bullfrog Rana catesbeiana (Suzuki et al., 1997). In those cases, differences are seem to be caused by the action of gonadal steroids [substance P: (DePalatis et al., 1985)/ galanin: (Kaplan et al., 1988)]. In the same way, differences of AM and PAMP expression between male and female specimens could be also explained by the action of sex steroids on AM and PAMP biology (Cameron et al., 2002; Pewitt et al., 1999). Given that the cells of the anterior pituitary lobe express both estrogen and androgen receptors (Mitchner et al., 1998; Pelletier, 2000), it may be possible a regulation of the preproAM-derived peptides production in pituitary due to sex hormones. It is known that in amphibians plasma levels of sex steroids change according to the annual reproductive cycle (Gobbett et al., 1991; Itoh et al., 1990; Mendonca et al., 1985), and therefore would be interesting to study the correlation between sex hormones and AM and PAMP production during the seasonal cycle of R. perezi.

**Pars tuberalis.** Endocrine cells of the frog pars tuberalis are immunoreactive for PAMP, but not for AM, which may also suggests—as in pars distalis—differential processing for AM and PAMP in amphibian pituitary. The significance of the immunostaining for PAMP in the pars tuberalis of frog adenohypophysis is unclear. Given that the pars tuberalis has received little attention in comparison to the other regions of the adenohypophysis, there are no studies about the presence of other regulatory peptides in the pars tuberalis of amphibians. Recently, however, great interest has come to the study of this region of the pituitary, because pars tuberalis is supposed to be involved in the transmission of photoperiodic stimuli to endocrine targets (Wittkowski et al., 1999). On one hand, studies in mammals have demonstrated a high density of melatonin receptor in the specific cells of the pars tuberalis (Barrett et al., 1999), and photoperiod has been reported to cause changes in their morphology and secretory activity (Bockers et al., 1995; Bockmann et al., 1996). On the other hand, the secretory products of pars tuberalis cells are able to regulate PRL release (Morgan et al., 1996). In amphibians, seasonal changes of the ultrastructure of the pars tuberalis have been described (Vandenbergh et al., 1973), suggesting that pars tuberalis may exert a similar function to that of mammals. It might be possible that PAMP produced by the pars tuberalis collaborates in this action.

### 4.3. AM in the neurohypophysis

In frog neurohypophysis, immunoreactivity for AM but not for PAMP has been found. AM immunoreactivity has been located in nerve fibers along the median eminence, pars nervosa and pars intermedia. In mammals, AM immunoreaction in neurohypophysis was previously observed (Hwang and Tang, 1999; Montuenga et al., 2000). These immunoreactive fibers seem to be axonal projections of AM neurons coming from the hypothalamus across the hypothalamo-neurohypophysial tract (Ueta et al., 1999). AM positive neurons are concentrated in the supraoptic and paraventricular lobes, where AM colocalize with both vasopressin and oxytocin (Ueta et al., 1995). Intracerebroventricular administration of AM stimulates the secretion of oxytocin by activating hypothalamic oxytocin-secreting neurons (Ueta et al., 2000).

In R. perezi, there exists a general study on the expression and distribution of AM in the central nervous system (Muñoz et al., 2001b) and a more detailed study in the hypothalamus (Muñoz et al., 2001a). This study has described six AM immunoreactive cell groups in the hypothalamus, with a light degree of colocalization with vasotocin, as well as AM immunoreactive axons that reached the median eminence to progress into the neural and intermediate lobes of the hypophysis. Our observations confirm this data and add more information. The finding in frog of a high AM immunoreactivity in the outer zone of median eminence, where the portal vessels are located, may be a clue about the function of neuronal AM in hypothalamo–hypophyseal system. AM could be secreted from the nerve fibres at this level and act as a hypophysiotropic factor, contributing to modulate the secretion of endocrine cells of the pars distalis.

The projection of AM immunoreactive nerve fibers into the pars intermedia may suggest another possible
function for AM, the involvement in regulation of MSH secretion. In amphibians, melanotrope cells of the *pars intermedia* are innervated by both aminergic and peptidergic fibers, some of them originating from different nuclei of the hypothalamus (Roubos, 1997). The neurotransmitters and regulatory peptides transported by those fibers contribute in the control of melanotrope activity and, consequently, in MSH secretion and skin colour adaptation (Vaudry et al., 1999). The presence of AM immunopositive neurons in the hypothalamic centers involved in this control, and AM immunoreactive fibres found in the *pars intermedia*, suggests that AM could be collaborating in MSH release regulation.

In summary, our data demonstrate that the two preproAM-derived peptides, AM and PAMP, have an important presence in the pituitary of amphibians, as already shown in mammals. The present findings suggest that AM and PAMP could be implicated in the regulation of frog pituitary function, although further studies are needed to evaluate their exact role of importance in pituitary physiology.

Acknowledgments

This work is supported by PIUNA (University of Navarra). We wish to thank Prof. F. Cuttitta (National Cancer Institute, Rockville, Maryland, USA) for the gift of antisera used in this work and Dr. E. Zudaire for help in the image analysis study.

References


López, L.J., Cuesta, N., Martínez, A., Montuenga, L., Cuttitta, F., 1999b. Proadrenomedullin N-terminal 20 peptide (PAMP) immu-


