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THE OCCURRENCE AND DISTRIBUTION OF ADRENOMEDULLIN (AM) IN THE ENDOCRINE PANCREAS OF SEVERAL POIKILOTHERM VERTEBRATES: AN IMMUNOHISTOCHEMICAL STUDY

Ioana TRANDABURU[1], Tiberiu TRANDABURU[2]
[1] Centre of Cytobiology, Institute of Biology, Bucharest, Romania
[2] University of Pitesti, Romania, Faculty of Sciences, Laboratory of Histology and Embryology

ABSTRACT. The pancreas of six amphibian and two turtle species was investigated for the immunohistochemical detection of the adrenomedullin (AM). The study revealed various degrees of labelling for this neuropeptide depending on the species and endocrine cell type differences, even within the same endocrine cell population. The immunolabelled neural structures represented by nerve fibbers and possible ganglionic perikarya or endocrine cells showed peri-insular localizations in the species Hyla arborea, whereas in the genus Rana were distributed throughout the organ. The AM distribution related to the main insular hormones (PP, SOM, INS, GLUC) was investigated on adjacent serial sections in the species Rana esculenta. The investigations have showed that only part of cells labelled for AM contained also pancreatic hormones in the following order: GLUC > SOM > PP > INS. The above findings and their functional significances supporting the good conservation of AM during phylogeny are discussed in connection with the results previously reported in mammals and other poikilotherm vertebrates.

Keywords: adrenomedullin, AM, amphibians, turtles, pancreas, immunohistochemistry

INTRODUCTION

Adrenomedullin, an α-amidated 52-aminoacid peptide, has been relative recently isolated from human pheochromacytoma by Kitamura et al., 1993. Based on the structural homologies of its molecule with calcitonin-gene related peptide (27%-CGRP), islet amyloid polypeptide (31%-IAPP), calcitonin (24%-C) and the B chain of insulin (34%-INS), on the overlapped biological effects and on the cross-reactivity of specific receptors, is considered at present that AM belong to the "insulin superfamily" of peptides encoded by genes located on chromosomes 11 and 12 (Wimalawansa, 1977; see also Olaru, 2005).

The initial biological effect ascribed to AM was that of vasorelaxant (Ishiyama et al., 1993). Subsequent investigations have characterized AM as a multifunctional peptide involved in many physiological actions like vasodilatation, both in the systemic circulation (Nuki et al., 1993) and in the pulmonary vascular bed (Nossaman et al., 1995), bronchodilatation (Kanazawa et al., 1994), regulation of renal functions (Ebara et al., 1994; Jougasaki et al., 1995), neurotransmission (Allen and Ferguson, 1996), growth stimulation (Miller et al., 1966) and defense against microorganisms (Walsh et al., 1998). Other investigations have demonstrated that AM is able to regulate the release of catecholamines (Kato et al., 1995), ACTH (Samson et al., 1995) and aldosterone (Mazzocchi et al., 1996). The above actions of this neuropeptide and of the related peptides are mediated by a family of membrane receptors coupled with G protein (Ishizaka et al., 1994). The specific receptor for AM has been cloned and sequenced since 1995 (Kaspas et al., 1995) and it has been isolated from the rat pancreas, where is expressed by all the islet cell types during embryogenesis (Martinez et al., 1996; 1998).

The biochemical and immunohistochemical investigations have established the occurrence of AM in various organs and tissues of certain mammalian species (dog, mouse, rat, swine, man), in both normal (Washimine et al., 1996; Mulder et al., 1996; Sakata et al., 1998; Asada et al., 1999; Lopez and Cuesta, 2002; Marutsuka et al., 2003 a.o) and pathological conditions (Satoh et al., 1995; Miller et al., 1996; Pio et al., 2001). As compared to mammals, only few references on this neuropeptide immunodetection in the organs of poikilotherm vertebrates are available at present (Gonzales et al., 1998; Lopez et al., 1999; Trandaburu et al., 2000; Collantes et al., 2003).

The present immunocytochemical investigations, carried out on the pancreas of altogether 8 species of amphibians and reptiles, have in view the demonstration of the good phylogenetic conservation of AM, the evolution of this neuropeptide occurrence in endocrine and/or neural elements of the organ and also its distribution related to the main endocrine cell types in the anuran species *Rana esculenta*.

MATERIALS AND METHODS

Animals

Adult specimens of amphibians and reptiles, of both sexes, captured in spring-time (April-May) from the surroundings of Bucharest, were kept unfed under

***Correspondence:** Ioana Trandaburu, Institute of Biology, Spl. Independenţei 296, 060031, Bucharest,
e-mail : itrandaburu@yahoo.com; ioana.trandaburu@ibiol.ro
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suitable laboratory conditions (in fresh-water aquaria and in terraria). The species and the number of specimens (in brackets) employed are listed below:

Amphibians

Urodela	newts:	*Triturus vulgaris* (4)
		Triturus cristatus (3)
Anura	frogs:	*Hyla arborea (3)*
		Rana temporaria (4)
		Rana esculenta (5)
		Xenopus laevis (4 specimens purchased from a commercial source)

Reptiles

	turtles:	*Emys orbicularis* (4)
		Testudo graeca (2)

Tissue preparation

All the animals were killed under chloroform anesthesia and the target organ (pancreas) was removed. Fragments or even the entire organ (amphibian pancreas) were immersed in Bouin's fluid for 24-36 h, dehydrated in increased concentrations of ethanol, cleared with toluene and paraffin-embedded. Serial sections of 6µm-thicness, prepared on a sledge microtome, were mounted apart on poly-L-lysine (Sigma, USA)-coated slides. The cellular co-localization of AM with the main insular hormones (PP, SOM, INS, GLUC) has been investigated on adjacent sections in the species *Rana esculenta*.

Primary antiserum

The primary antibody – polyclonar rabbit anti-goat adrenomedullin – was kindly provided by Prof. R.E.Lang, Institute of Physiology, Marburg, Germany.

Immunohistochemical protocol

The deparaffinized and rehydrated sections were treated according to the peroxidase anti-peroxidase (PAP) technique (Sternberger, 1974) modified as follows:

- the second and third incubation steps in the original protocol were replaced with donkey anti-rabbit IgG peroxidase linked the whole antibody produced by Amersham Pharmacia Biotech, UK.

-an incubation in ammonium Ni-sulphate (Riedel-de Haën, Germany), prior to the development of immunoreaction in 3,3'-diaminobenzidine tetrahydrochloride (DAB), was inserted for the enhancement of the specific reaction.

The 1:400 and 1:500 concentrations of the primary antiserum which maximally stains the immunoreactive structures without any other unspecific reaction were chosen. The sections were finally dehydrated in ethanol, cleared with xylene, mounted in Entellan (E.Merck, Germany) and examined in a Zeiss (Oberkochen, Germany) Photomicroscope II.

Specificity controls

The specificity of the immunoreaction was tested by replacing the primary antibody and donkey anti-rabbit IgG peroxidase linked antiserum with phosphate saline (PBS) or with Tris-saline (TBS) buffers. The specificity of the primary antibody was tested by preadsorbtion (24h at 4oC) with the corresponding antigen (human AM 1-52, Peninsula Labs., Germany) or with heterologous, but structurally related antigens (CGRP, C, IAPP).

RESULTS AND DISCUSSIONS

The optimal immunostaings were obtained by using the dilution 1:400 of the primary antiserum for the anura genera Rana and Xenopus (Figures. 4, 5, 6a-9a) and 1:500 for both species of newts and turtles under study and for the frog species *Hyla arborea* (Figures. 1-3, 10, 11). With these dilutions, which didn't allow an unspecific reaction (background), the immunostaining revealed reactive sites not only in the endocrine and exocrine pancreas but also in the intrinsic nerve fibers of this gland. Finally, should be mentioned the observations that AM didn't seem to label the secretory granules matrix of endocrine cells, as well as the high degree of interspecies heterogeneity of the staining.

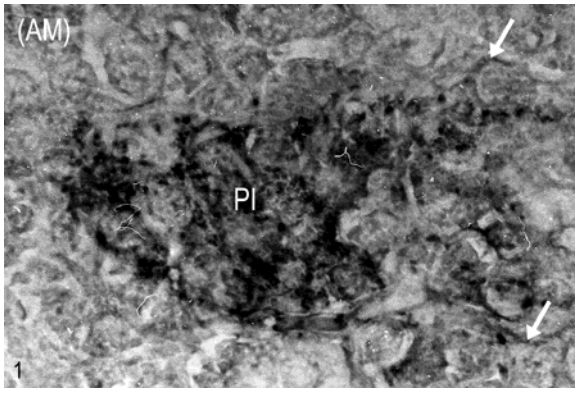

Fig. 1 Picture showing a pancreatic islet of Langerhans in the newt species *Triturus vulgaris* immunostained for AM. Several peri-acinar nerve fibers (arrows) can be also seen. PAP-procedure, x690

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Newts

The diffuse organization of endocrine pancreas as small clusters of cells with islet-like appearance and single cells randomly disseminated in the exocrine tissue render the localization of AM-immunostained cells difficult. In addition, great variations of the immunostaining intensity among the reactive endocrine cells could be also noticed (Fig. 1). Besides the labelled cells, a lot of AM-immunopositive fibbers showing peri-vascular and peri-acinar distributions were frequently observed (Fig. 2).

Frogs

In spite of the close similarities regarding the general organization and cellular lay-out of the endocrine pancreas, quite different patterns of immunoreactivity for AM in the genera Hyla, Rana and Xenopus were recorded. Thus, in the species *Hyla arborea* only few peri-insular nerve fibers and possible endocrine cells appeared strongly immunoreactive for this neuropeptide (Fig. 3). Unlike this species, many endocrine cells and a small amount of fine nerve fibers spread in the exocrine tissue of the genera Rana and Xenopus could be observed (Figures. 4, 5). In addition, the co-localization investigations carried-out on the pancreas of *Rana esculenta* showed that only a few AM labelled cells (Figures. 6a-9a) contained glucagon (Fig. 6b), insulin (Fig. 7b), pancreatic polypeptide (Fig. 8b) or somatostatin (Fig. 9b) in adjacent serial sections.

The investigations have revealed the presence of several minority subpopulations of A, B, PP and SOM cells exhibiting also AM. Without any pretention of exact quantitative evaluation, they indicate the following order of AM co-localizations with pancreatic hormones: GLUC > SOM > PP > INS.

Turtles

The very loose organization of the endocrine pancreas in both reptile species under study (*Emys orbicularis* and *Testudo graeca*) suggesting the primitive structures of this gland, makes the co-localization studies of AM-immunoreactivity more difficult than in anurans. In addition to this difficulty, the small size of the endocrine cells and the relatively high background staining of the acinar tissue should be also taken into account. A relatively large number of singular AM-immunoreactive cells were found disseminated throughout the pancreas of both turtle species (Figures. 10, 11). As a rule, they appeared strongly immunostained with 1:500 dilution of the primary antibody, which revealed also a rich network of fine, weakly labelled nerve fibers, spread uniformly throughout this gland (Figures. 10, 11).

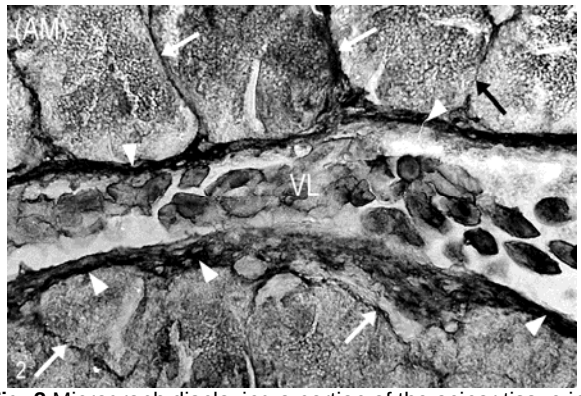


Fig. 2 Micrograph displaying a portion of the acinar tissue in the newt *Triturus cristatus* immunolabelled for AM. To observe the peri-acinar (arrows) and peri-vascular (arrowheads) distributions of nerve fibers. VL= vascular lumen. PAP-procedure, x445

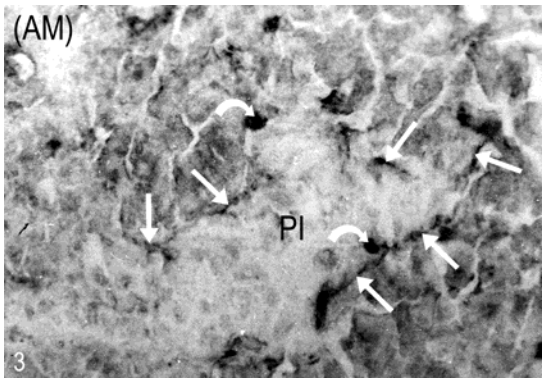
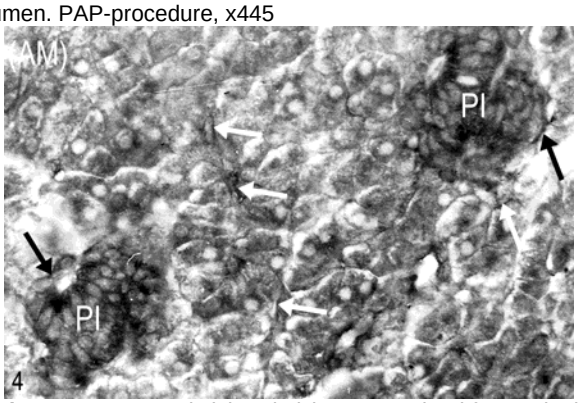
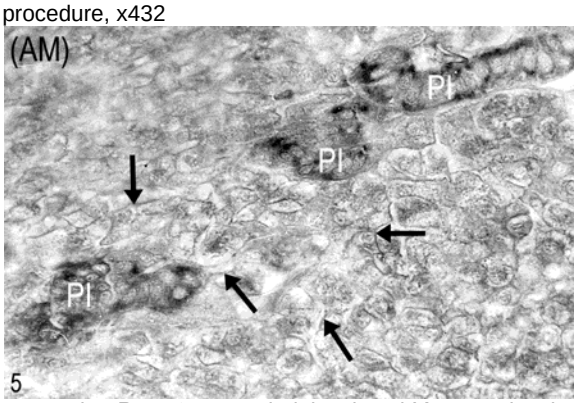
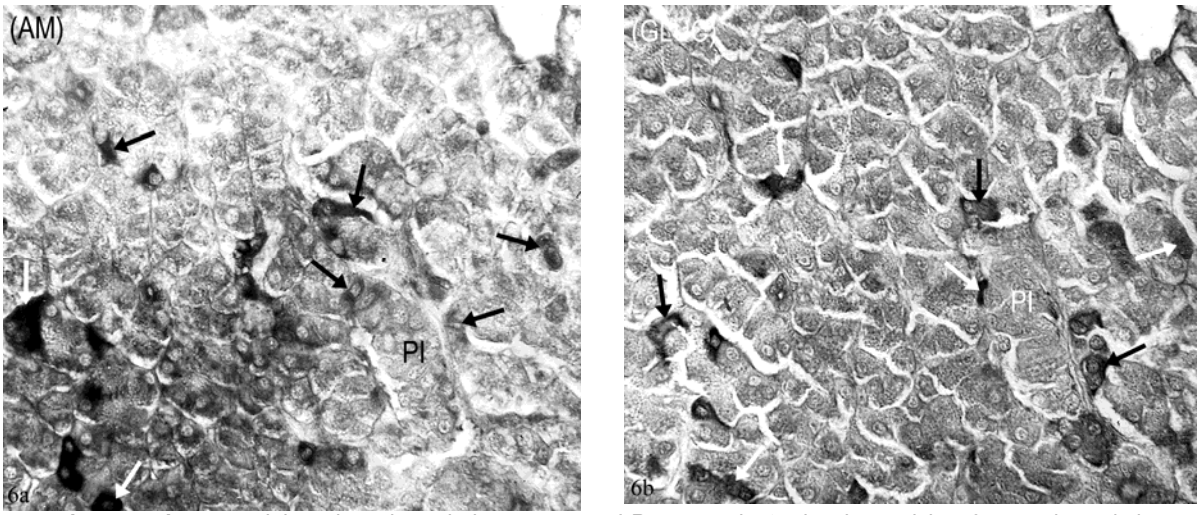


Fig. 3 Peri-insular nerve fibers (arrows) and possible ganglionic perikarya or endocrine elements (curved arrows) immunopositive for AM detected in the pancreas of the frog *Hyla arborea*. PI = pancreatic islet. PAP-procedure, x432

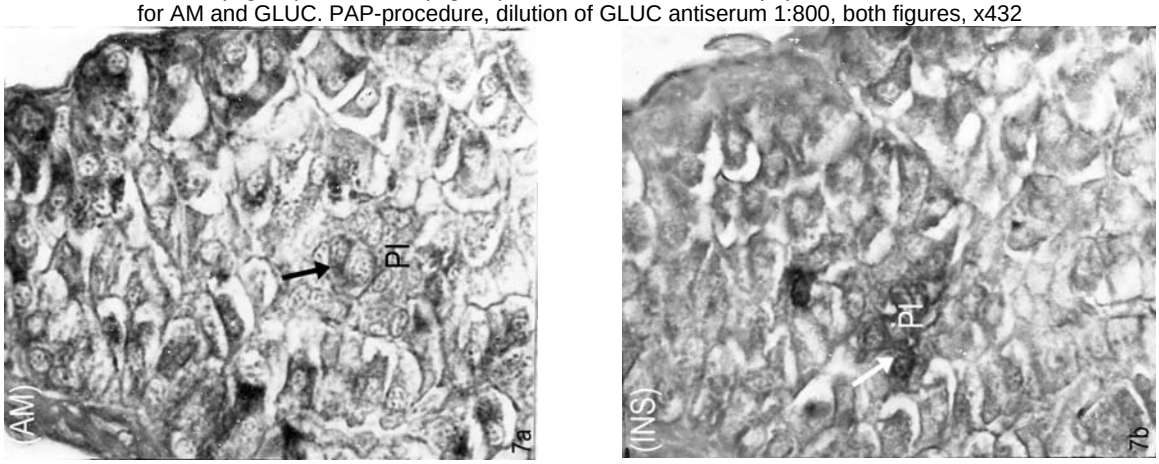




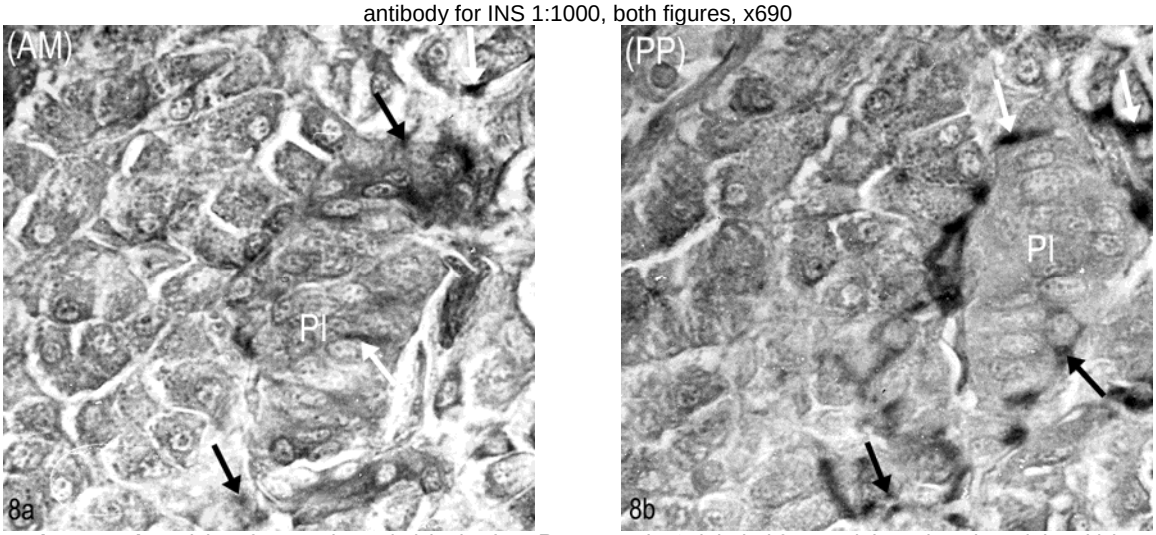
Figs. 4, 5 Pancreatic islets (PI) immunostained for AM in the frog species *Rana temporaria* (Fig. 4) and *Xenopus laevis* (Fig. 5). To note the peri-acinar profiles (arrows) of the gland intrinsic innervation. PAP-procedure, both figures, x432



Figs. 6a, 6b Two serial sections through the pancreas of *Rana esculenta* showing an islet of Langerhans (PI) immunomarked for AM (Fig. 6a) and GLUC (Fig. 6b). The arrows indicate the population of endocrine cells labeled both for AM and GLUC. PAP-procedure, dilution of GLUC antiserum 1:800, both figures, x432



Figs. 7a, 7b A pancreatic islet (PI) in *Rana esculenta* immunostained for AM (Fig. 7a) and INS (Fig. 7b) in consecutive serial sections. An endocrine cell containing AM and INS is indicated by arrows. PAP-procedure, dilution of the primary antibody for INS 1:1000, both figures, x690



Figs. 8a, 8b An islet of Langerhans (PI) in the frog *Rana esculenta* labeled for AM (Fig. 8a) and PP (Fig. 8b) in consecutive sections. The endocrine cells exhibiting AM and PP are indicated by arrows. PAP-procedure, PP dilution 1:800, both figures, x690

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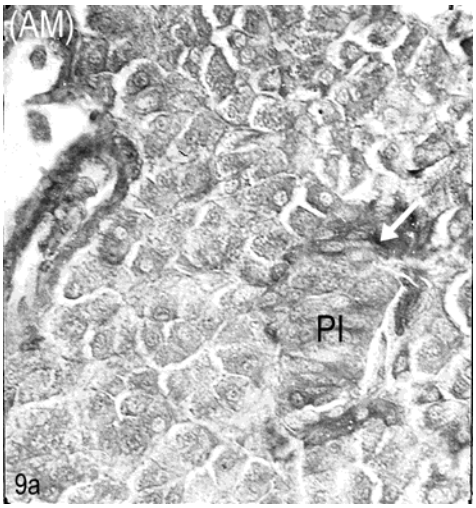
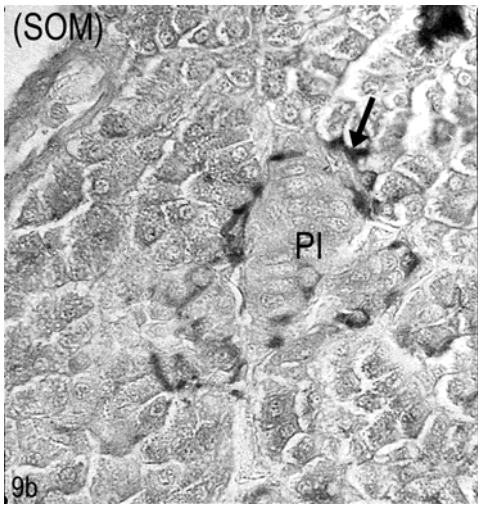
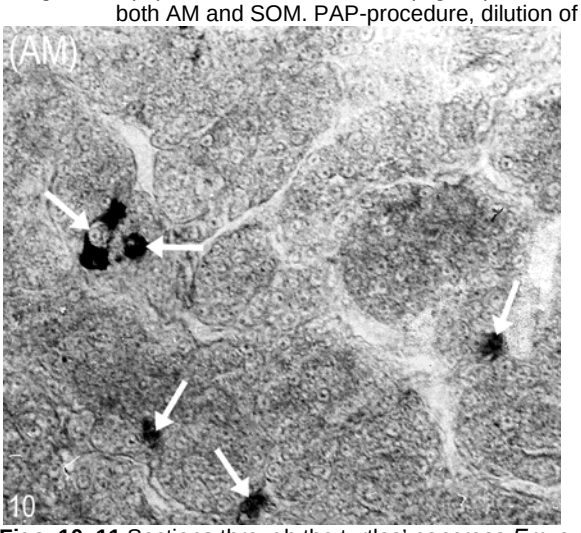
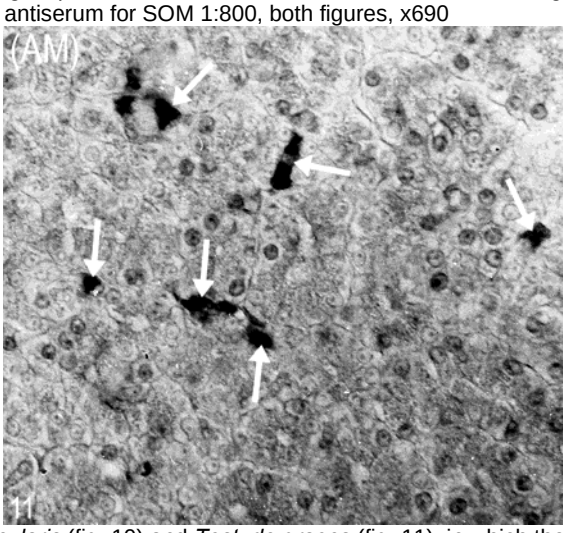




Figs. 9a, 9b Two consecutive sections through the pancreas of *Rana esculenta*, in which can be seen an islet of Langerhans (PI) immunolabelled for AM (Fig. 9a) and SOM (Fig. 9b). The arrows indicate an endocrine cell containing both AM and SOM. PAP-procedure, dilution of the antiserum for SOM 1:800, both figures, x690





Figs. 10, 11 Sections through the turtles' pancreas *Emys orbicularis* (fig. 10) and *Testudo graeca* (fig. 11), in which the arrows indicate the AM immunostained cells. PAP-procedure, both figures, x430

The present study is one of the few comparative approaches to the occurrence, topographic distribution and cellular co-localizations of AM in the pancreas of lower vertebrates. In addition, so far as we know, it reports for the first time the cellular distribution of this neuropeptide in the above organ of turtles. Thus, this study and others (Lopez et al., 1999; Ogoshi et al., 2003), support the well phylogenetic conservation of AM not only in the pancreas, but also in various glandular organs (Gonzales et al., 1998; Trandaburu et al., 2000; Collantes et al., 2003) of nonmammalian vertebrates. Finally, the identification of an AM-like immunoreactivity in the nervous system of the digestive tract of echinoderms (Martinez et al., 1996) has enlarged considerably our knowledge on its early phylogenetic origin. The isolation and sequencing of this molecule involved in the regulation of muscle movement and neurotransmission should show the exact homology ratio between echinoderm AM and its mammalian counterpart.

Coming back to the poikilotherm vertebrates, AM-immunoreactivity in the pancreas of the studied animals showed similar features to those reported in mammals but also several peculiarities. Among the similarities with mammals, the heterogeneous distribution of immunoreactivity for AM depends on the species and endocrine cell type differences, even within the same endocrine cell population. They represent widely spread features reported not only in the mammalian pancreas (Washimine et al., 1995; Montuenga et al., 1997; Martinez et al., 1998; Lopez and Cuesta, 2002), but also in other endocrine and neural tissues (Mulder et al., 1996; Sato et al., 1996; Sakata et al., 1998; Kitani et al., 1998; Collantes et al., 2003; Marutsuka et al., 2003).

A variety of reasons may be responsible both for interspecific and intercellular heterogeneity of the immunoreactivity for AM in the endocrine pancreas. Apart from the methodological reasons and those derived from the antibody specificities, is very possible

that the different degree of masking of AM epitope by the co-stored hormones within the secretory granules could be responsible for the various densities and even for the lack of immunostaining.

As was already pointed out, the occurrence and cellular distribution of AM, at least in the pancreas of amphibians, showed, in comparison with mammals, several peculiar features. One of them was the far more restricted occurrence of this neuropeptide in the pancreatic islets of *Hyla arborea,* as well as its low incidence in the B cells of the two species of the genus Rana, as compared with that reported during organ development in rats (Martinez et al., 1998) or in adult man (Lopez and Cuesta, 2002).

CONCLUSIONS

The paper represents one of the few immunohistochemical demonstrations of the occurrence, topographic distribution and co-localization of AM in the pancreas of poikilotherm vertebrates. Therefore its aim is to enrich our present knowledge on the presumed phylogenetic perenity of this neuropeptide in such glandular organ.

The co-expression of AM with the main insular hormones (PP, SOM, INS, GLUC) detected at least in a frog species (*Rana esculenta*), as well as its co-storing probably with other neurotransmitters in the organ acinar tissue, suggest the involvement in a large variety of physiological actions already demonstrated in mammals or only suggested in other species of poikilotherm vertebrates. Nevertheless, a series of questions regarding the co-storing and releasing mechanism of AM from the endocrine cell types claim answers in the future. Finally, it should be mentioned the necessity of promoting further investigations not only on the pancreas, but also on other organs of lower vertebrates in order to understand the releasing mechanism (s) of this multifunctional neuropeptide

As a matter of fact, as Lopez and Cuesta (2002) reported, AM has been found in embryonic mammalian pancreas from the earliest stages of the development co-localizing with all pancreatic hormones, although in adults only co-expression with PP was kept. Considering the aforesaid, our findings in the species *Rana esculenta* regarding the order of AM-co-localizations with the main subpopulations of hormones producing cells, seem to be plausible and this so much the more the endocrine pancreas of the adult frog, unlike to that of mammals, displayed the neuropeptide in all endocrine cell types.

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