

# THE IMMUNE-ULTRASTRUCTURAL INVESTIGATIONS ON ADRENOMEDULLIN (AM) DISTRIBUTION IN THE PANCREAS OF AMPHIBIAN *RANA ESCULENTA*

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## ABSTRACT

The pancreas of the frog *Rana esculenta* was investigated in electron microscopy for the immunocytochemical detection of the adrenomedullin (AM). These investigations revealed that AM didn't label the secretory granules matrix of all endocrine cells, but rather several minority subpopulations of AM, SOM, PP and INS cells. Concretely, the results indicated that 10%-15% from glucagon (GLUC) producing cells, 4%-8% from somatostatin (SOM) and pancreatic polypeptide (PP) cells and only 2%-5% from insulin (INS) cells contained also AM. The sites of antigen-antibody reaction, labelled with colloidal gold particles, have been detected in the cells cytoplasm, randomly distributed among the secretory granules, surrounding them, or overlapped on the hormonal cores. In the exocrine pancreas, reactive nerve fibers to AM occurred distributed among acini, in the ducts walls, or in those of blood vessels. The above findings and their functional significances are discussed in connection with the results previously reported in mammals and other lower vertebrates.

**KEY WORDS:** adrenomedullin, AM, frog, pancreas, immunocytochemistry

## INTRODUCTION

An  $\alpha$ -amidated 52-aminoacid peptide with multiple biological effects (Trandaburu et al., 2000; Trandaburu and Trandaburu, 2010), named adrenomedullin (AM) has been recently isolated from human pheochromocytoma by Kitamura et al., 1993. The structural homologies of its molecular structure with calcitonin-gene related peptide (27%-CGRP), islet amyloid polypeptide (31%-IAPP), calcitonin (24%-C) and the B chain of insulin (34%-INS), as well as the overlapped biological effects and the cross-reactivities of specific receptors make it possible today to state that AM belongs to the "insulin superfamily" of peptides (Wimalawansa, 1977; see also Olaru, 2005).

The first biological effect ascribed to AM was that of vasorelaxant (Ishiyama et al., 1993). This multifunctional peptide appeared also involved in a lot of physiological actions like hypotension (Nuki et al., 1993), neural transmission (Allen and Ferguson, 1996), bronchodilatation (Kanazawa et al., 1994), growth stimulation (Miller et al., 1996), and defense against microorganisms (Walsh et al., 1998). The last but not the least AM regulates the release of catecholamines (Kato et al., 1995), aldosterone (Mazzochi et al., 1996), ACTH (Samson et al., 1995) and also the modulation of insulin secretion (Mulder 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 previous biochemical and immunohistochemical investigations have demonstrated the occurrence of AM in various organs and tissues of mammals (man, swine,

mouse, rat, dog) in both normal (Washimine et al., 1995; Mulder et al., 1996; Sakata et al., 1998; Asada et al., 1999; Lopez and Cuesta, 2002; Marutsuka et al., 2003 a.o) and pathological states (Satoh et al., 1995; Miller et al., 1996; Pio et al., 2001). As compared to mammals only few references on the immunodetection of this neuropeptide in the organs of poikilotherm vertebrates are available at present (Gonzales et al., 1998; Lopez et al., 1999; Trandaburu et al., 2000; Trandaburu and Trandaburu, 2010). Considering the afore said, the present immunoultrastructural investigations, carried out on the anuran species *Rana esculenta*, have in view the highlighting of AM co-existence with the main insular hormones and/or with the pancreatic nerve fibers.

## MATERIALS AND METHODS

### Animals

Four adult specimens of *Rana esculenta* (L) of both sexes, captured in the spring-time (April-May) from the surroundings of Bucharest, were kept unfed in fresh-water aquaria for 1-3 days.

### Tissue preparation

The animals were killed under chloroform anesthesia and the target organ was removed. The pancreatic tissue was cut into small slices ( $\leq 2\text{mm}^3$ ) and fixed by immersion in a cold Zamboni's solution (2% paraformaldehyde and 0.5% picric acid in 0.1M phosphate buffer, pH 7.2; Zamboni and de Martino, 1967). The pieces were embedded in Lowicryl K4M according to Carlemalm et al., 1982.

The ultrathin sections for the immunoelectron microscopic investigations, prepared on a Reichert OmU2 ultramicrotome, were collected on pioloform-coated Ni-grids. Without trimming of Lowicryl K4M, the ultrathin sections were directly exposed to the primary antibody containing 0.25% serumalbumin. After incubation period of 4h at room temperature and subsequent proper rinsing, the grids were transferred to a protein A-gold solution (1:25) for 1h prepared according to the method of post-inclusion developed by Roth (1982) and Bendayan (1984). The diameters of protein A-gold particles of immunogold conjugates produced by British Biocell International (Cardiff, UK) were of 5.8 nm. The prepares were stained in accordance with the protocol of Reynolds (1963) and observed with a Philips electron microscope at 80 kV (Philips EM 201).

#### Primary antibody

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

#### Specificity controls

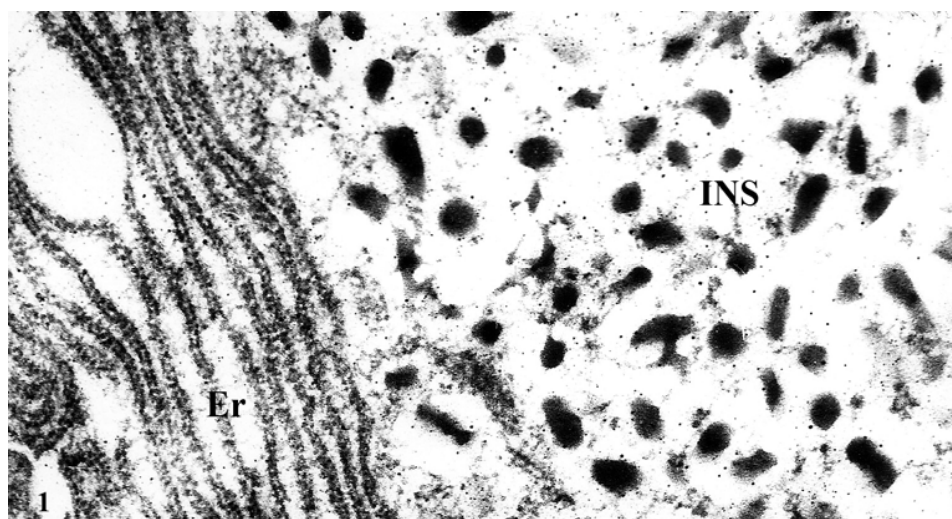
The controls for the specificity of the immunoreaction were obtained by using adequate buffer (phosphate saline-PBS or Tris-saline-TBS) instead of anti-goat adrenomedullin serum, goat anti-rabbit IgG or PAP-complex, respectively. Furthermore, the anti-adrenomedullin serum was tested for nonspecific staining after absorption to a solid phase affinity matrix of adrenomedullin conjugated to cyanogen-bromide activated Sepharose (Pharmacia, Uppsala, Sweden). Cross-reactions to guinea pig antiovine INS serum (Linco Res Inc, St Charles, Mo, USA), rabbit polyclonal antibody to GLUC (Biotrend Chem GmbH, Köln, Germany), rabbit polyclonal antibody to SOM (Biotrend

Chem GmbH, Köln, Germany) or guinea pig anti-rat PP (Linco Res Inc, St Charles, Mo, USA) were not observed.

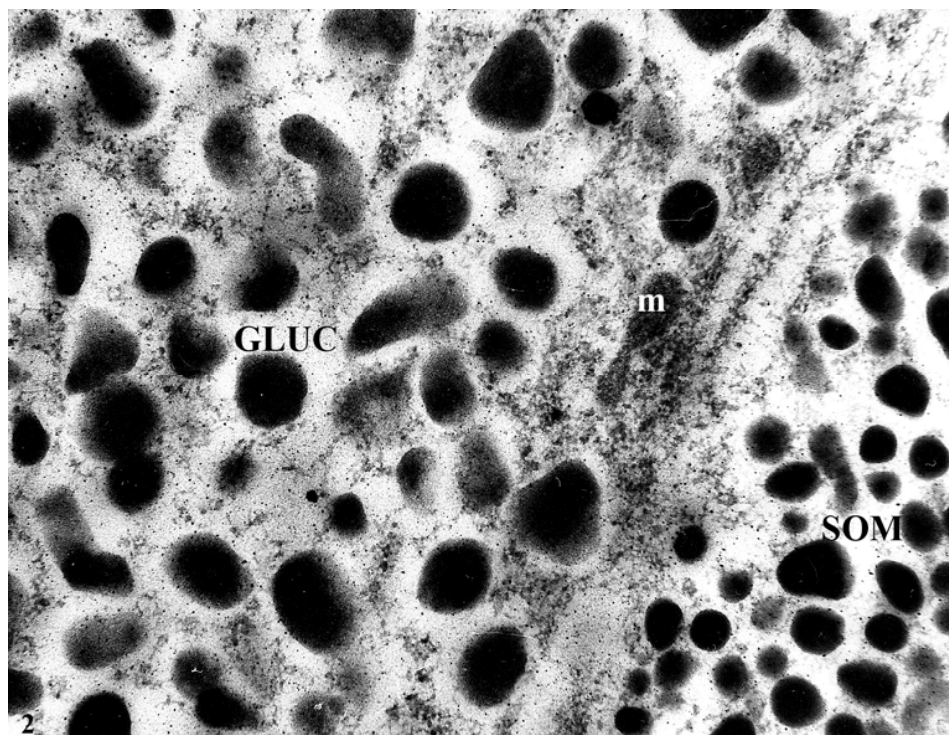
## RESULTS AND DISCUSSIONS

The optimal immunostaining of the frog pancreas was obtained by using the dilution 1:300 of the primary antiserum, which didn't allow an unspecific reaction (background). With the above dilution the immunostaining revealed reactive sites not only in the pancreatic islets, but also in the intrinsic nerve fibers of the gland. In addition, should be mentioned that AM didn't seem to label the secretory granules matrix of many endocrine cells. In this meaning our investigations showed that only a few AM labelled endocrine cells contained also glucagon, insulin, somatostatin or pancreatic polypeptide hormones. Finally, the investigations have revealed the existence of several minority subpopulations of A, B, PP and SOM cells exhibiting AM. Without any pretention of exact quantitative evaluation, they indicated that 10%-15% from glucagon (GLUC) producing cells, 4%-8% from somatostatin (SOM) and pancreatic polypeptide (PP) cells and only 2%-5% from insulin (INS) cells contained also AM.

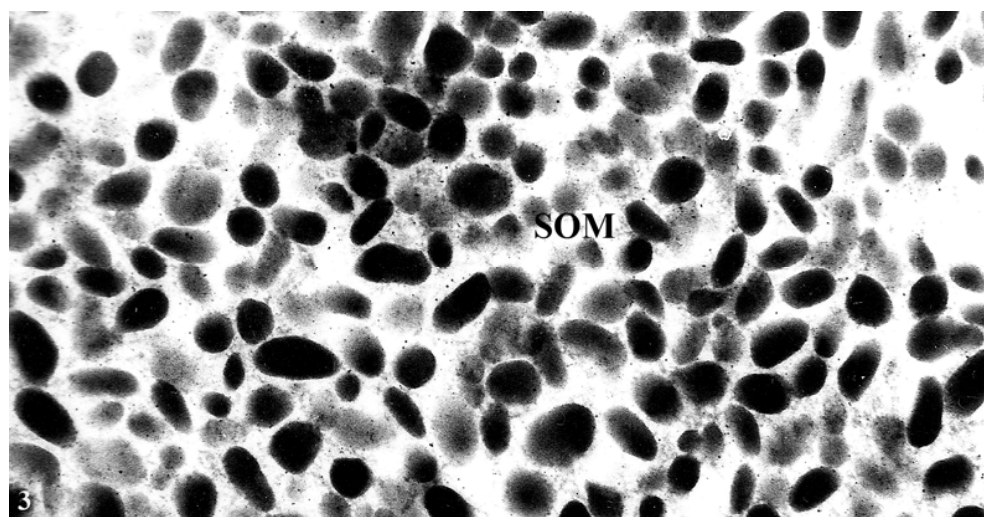
The sites of antigen-antibody reaction, labelled with colloidal gold particles of 5.8nm diameters, have been detected in the cytoplasm of reactive cells, randomly distributed among the secretory granules (Figures 1- 4), surrounding them or overlapped on the hormonal content (Figures 1- 4,6). The same distributions of colloidal Au particles were found also in the nerve fibers penetrating the gland (Fig.6). In the exocrine pancreas, AM immunopositive nerve fibers showed peri-acinar and peri-vascular distributions.



**Fig.1** Picture showing an area from an insulin (INS) cell at the limit with exocrine pancreas. The AM localization in the cytoplasm of cell can be seen; Er = ergastoplasm; 1:300 dilution of the primary antibody; Protein-A gold labelling; Post-inclusion method; 78, 340.

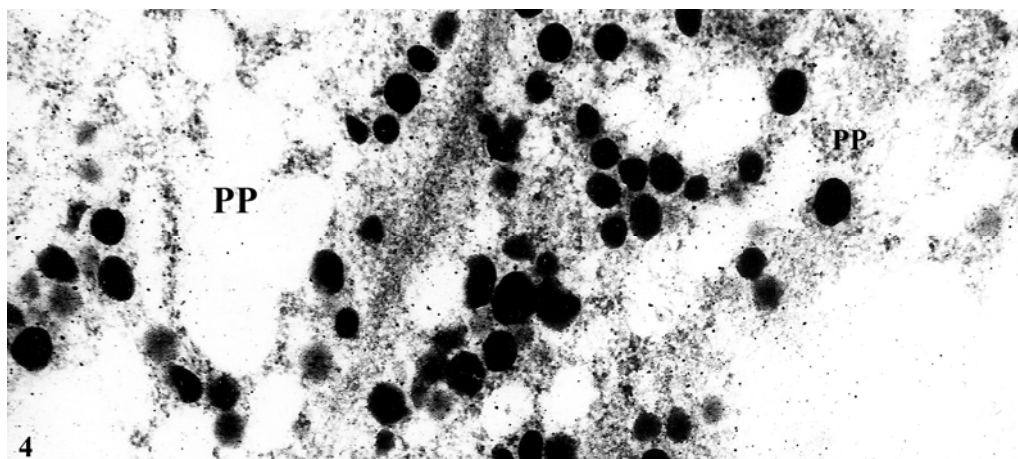


**Fig.2** Detail from two adjacent cells producing glucagon (GLUC) and somatostatin (SOM). The immunoreaction for AM is spread randomly in cytoplasm and more rarely on the secretory granules cores; m = mitochondria; 1:300 dilution of the primary antiserum; Protein-A gold labelling; Post-inclusion method; x89,000.

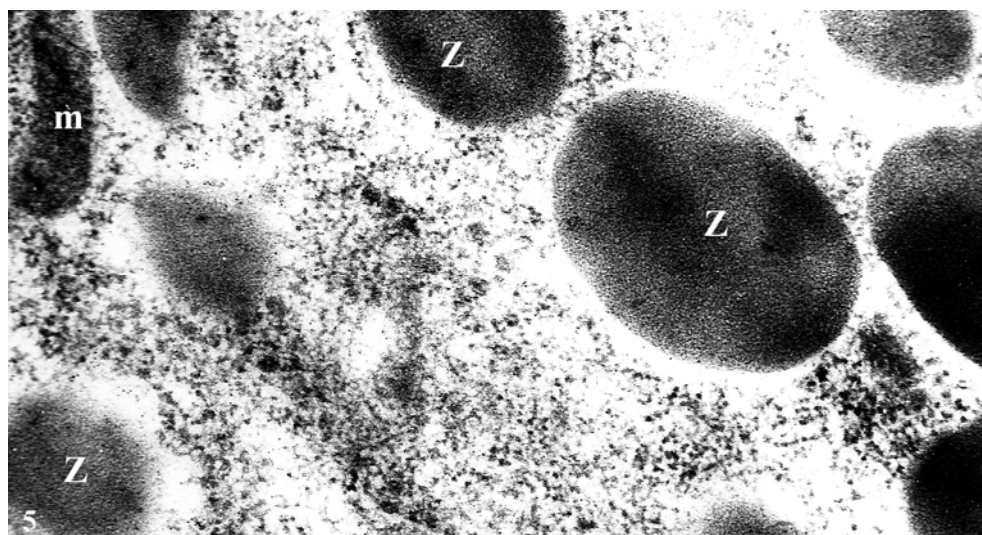


**Fig.3** Portion of a somatostatin (SOM) cell. Immunoreactive sites for AM occur localized randomly among the

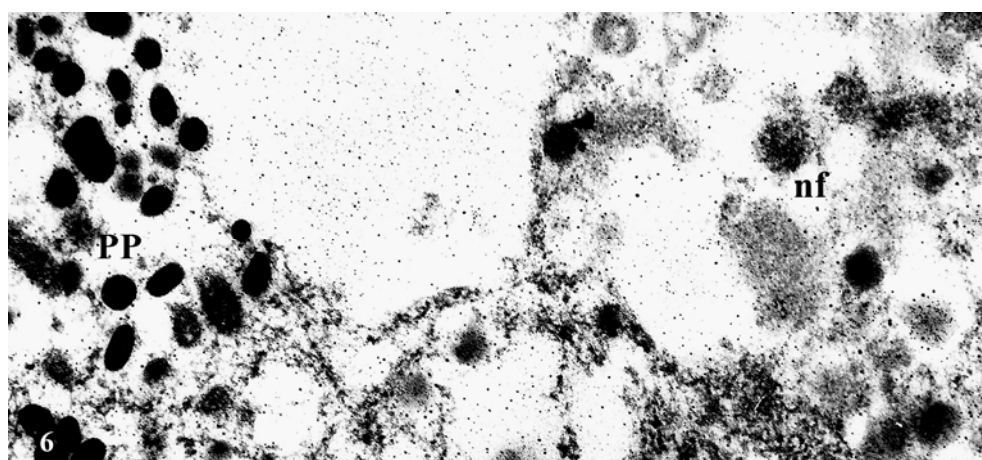
**Fig.3** Portion of a somatostatin (SOM) cell. Immunoreactive sites for AM occur localized randomly among the secretory granules and also at their periphery. Dilution of the primary antibody 1:300; Protein A-gold reaction; Post-inclusion method; x90,000.



**Fig.4** Adjacent pancreatic polypeptide (PP) cells showing the AM immunostaining randomly distributed in the cytoplasm of both cells and more rarely on the cores of secretory granules. 1:300 dilution of the primary antiserum; Protein A-gold labelling; Post-inclusion method; x88,300.



**Fig.5** Area of an exocrine cell devoid of immunoreaction for AM; m = mitochondria; Z = zymogen granules; Dilution of the primary antibody 1:300; Protein A-gold labelling; Post-inclusion method; x88,300.



**Fig.6** Portion of a pancreatic polypeptide (PP) cell and of pancreatic nerve fibers immunostained for AM. The reactive sites occurred distributed randomly in the cytoplasm and neuroplasm of the immunomarked structures and relatively less at their periphery; nf = nerve fibber; Dilution of the primary antibody 1;300; Protein A-gold reaction; Post-inclusion method; x19,000.

The present study is one of the few approaches to the occurrence, topographic distribution and subcellular co-localizations of AM in the pancreas of lower vertebrates. Thus, this study and others (Lopez et al., 1999; Ogoshi et al., 2003; Trandaburu and Trandaburu, 2010) 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 AM-like 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 lower vertebrates, AM immunoreactivity in the pancreas of *Rana esculenta* showed not only similar features to those reported in mammals but also several peculiarities. Among the similarities with mammals should be mentioned the fact that AM distribution depends on the differences among endocrine cell types, even those recorded 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 for the intercellular heterogeneity of the immunoreactivity for AM in the endocrine pancreas of the anuran studied. Apart from the methodological reasons and those derived from the antibody specificities, it is very possible that the different degree of masking of AM epitope by the co-stored neuropeptides within the secretory granules could be responsible for the various densities and even for the lack of immunostaining.

As was previously mentioned, the ultrastructural distribution of AM, at least in the pancreas of *Rana esculenta*, showed, in comparison with mammals, several peculiar features. One of them was the far more restricted occurrence of this neuropeptide, as well as its low incidence in the B cells of this anuran species, as compared with that reported during organ development in rats (Martinez et al., 1998) or in adult man (Lopez and Cuesta, 2002).

As a matter of fact, as Lopez and Cuesta (2002) reported, AM has been detected in embryonic mammalian pancreas from the earliest stages of the development co-localized with all pancreatic hormones, although in adults only the co-expression with PP cells 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 adult frog, unlike to that of mammals, displayed the neuropeptide in all endocrine cell types.

Several physiological roles of AM have been advanced by Lopez and Cuesta (2002) in mammals, including the man. According to these authors, the neuropeptide inhibits the insulin secretion of B cells. In relation to another physiological role clinical data showed that AM, which is raised in some groups of both types I and II diabetic patients, might have triggered the disease in a subset of them. On the other hand, pancreatic cells containing AM are involved in the response to septic shock by increasing the circulating levels. Finally, AM may play a role in the growth and morphogenesis of the pancreas.

## CONCLUSIONS

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

The co-expression of AM with the relative restricted subpopulations of the main insular hormones (GLUC, SOM, PP, INS) detected in the frog *Rana esculenta*, as well as its co-storing with other neurotransmitters in the organ acinar tissue, suggest the involvement in a large variety of physiological actions already demonstrated in mammals or only presumed in other species of lower vertebrates. Nevertheless, many 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) and effects of this multifunctional neuropeptide.

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