Acta Histochemica 118 (2016) 213–218

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Localization of amylin-like immunoreactivity in melanocyte-stimulating hormone-containing cells of the pars intermedia but not those of the pars distalis in the axolotl (*Ambystoma mexicanum*) pituitary

Hirohumi Suzuki[a,*], Toshiharu Yamamoto[b]

[a] *Department of Biology, Fukuoka University of Education, Akamabunkyo-machi 1-1, Munakata, Fukuoka 811-4192, Japan*
[b] *Brain Functions and Neuroscience Unit, Department of Oral Science, Graduate School of Dentistry, Kanagawa Dental University, Inaoka-cho 82, Yokosuka, Kanagawa 238-8580, Japan*

ARTICLE INFO

Article history:
Received 2 August 2015
Received in revised form 6 January 2016
Accepted 6 January 2016

Keywords:
Amylin
IAPP
MSH
Pituitary
Axolotl
Amphibian

ABSTRACT

Immunohistochemical techniques were employed to investigate the distribution of amylin-like immunoreactivity in the axolotl (*Ambystoma mexicanum*) pituitary. Amylin-immunoreactive cells were observed in the pars intermedia, and these cells were found to be immunoreactive for α-melanocyte-stimulating hormone (αMSH) as well. In contrast, αMSH-immunoreactive cells in the pars distalis were immuno-negaitive for amylin. These light microscopic findings were confirmed by immunoelectron microscopy. Amylin-immunoreactive signals were located on the haloes of presumable secretory granules in association with αMSH-immunoreactive signals in the amylin-positive cells. However, in the pars distalis, the αMSH-positive cells did not contain amylin-immunoreactive secretory granules. Western blot analysis of axolotl pituitary extracts revealed the labeling of a protein band at approximately 10.5-kDa by the anti-rat amylin serum, which was not labeled by the anti-αMSH antibody. These findings indicate that amylin secreted from MSH-producing cells in the pars intermedia may modulate MSH secretion in an autocrine fashion and may participate in MSH functions such as fatty homeostasis together with MSH.

1. Introduction

Amylin, also termed as islet amyloid polypeptide (IAPP), is a bioactive peptide initially isolated from a human insulinoma and from islet amyloid deposits of the human and cat pancreas (Westermark et al., 1987; Cooper et al., 1988). Amylin is a member of the calcitonin related peptide family, which consists of calcitonin, α- and β-calcitonin gene-related peptides (CGRPs), adrenomedullin, and intermedin (Cao et al., 2013). In humans, amylin is derived from an 89-amino acid precursor and consists of 37 amino acids obtained by proteolytic processing (Sanke et al., 1988) during which, amylin is stored in mature secretory granules in pancreatic β cells (Marzban et al., 2005). Immunoelectron microscopy has revealed that amylin and insulin are co-localized in secretory granules in human β cells (Lukinius et al., 1989). Pharmacological experiments have demonstrated that amylin is co-secreted with insulin from cultured pancreatic cells as well as isolated rat pancreatic islets (Kahn et al., 1990; Stridsberg et al., 1993). By autoradiography, binding sites for amylin have been found at several locations discretely in the brains of rats and monkeys (Christopoulos et al., 1995). The distribution of these binding sites in the area postrema located outside the blood–brain barrier, suggests that the area postrema is a possible target for amylin produced by the pancreatic islets (Westermark et al., 2011). Molecular biological techniques have shown that amylin is widely conserved in vertebrates (Westermark et al., 2002, 2011; Martínez-Álvarez et al., 2008; Cao et al., 2013). In the goldfish (*Carassius auratus*), amylin mRNA has been detected in several tissues including the pituitary (Martínez-Álvarez et al., 2008). Although amylin is known to be localized in functionally unknown X-cells of the frog pancreas (Suzuki and Yamamoto, 2014b), detailed tissue distribution of the peptide in amphibians is unclear. In this study, we examined distribution of amylin in the pituitary of the axolotl (*Ambystoma mexicanum*) and observed its association with the melanocyte-stimulating hormone (MSH)-producing cells in the pars intermedia.

* Corresponding author. Fax: +81 940 35 1716.
E-mail address: spiderhs@fukuoka-edu.ac.jp (H. Suzuki).

http://dx.doi.org/10.1016/j.acthis.2016.01.001
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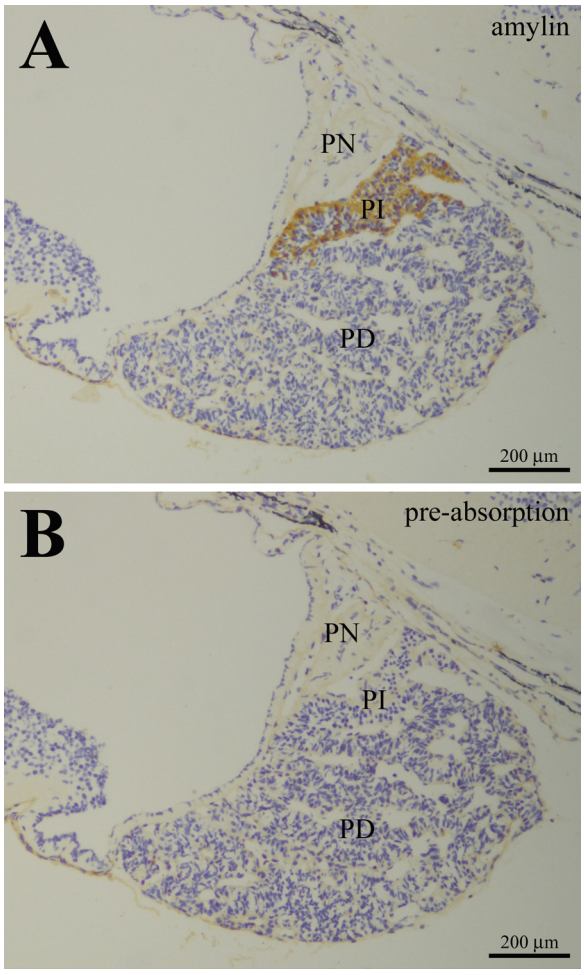

Fig. 1. Serial sections of a pituitary showing amylin-like immunoreactivity in the pars intermmedia (A). A section demonstrating no immunoreactivity for amylin by pre-absorption with synthetic amylin (B). Immunoreactivities are visualized by DAB deposition. Nuclei are stained with thionin. PD, pars distalis; PI, pars intermedia; PN, pars nervosa. Scale bars = 200 μm.

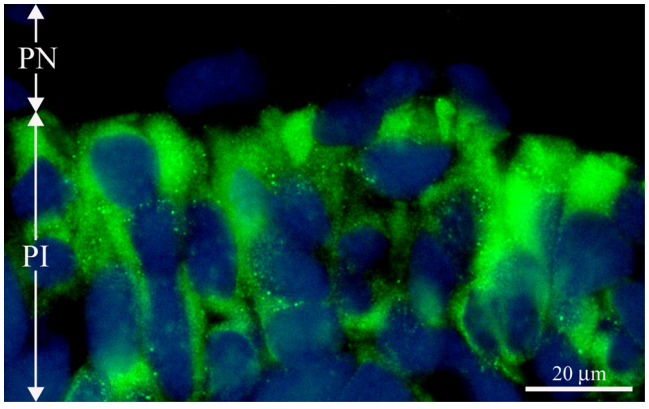

Fig. 2. Higher magnification of the section showing amylin-positive cells in the pars intermedia (PI) visualized by Alexa Fluor 488. Nuclei are stained with DAPI. PN, pars nervosa. Scale bar = 20 μm.

2. Materials and methods

2.1. Animals

Twenty-nine albino axolotls (*A. mexicanum*) of both sexes (body length, 19–22 cm) were obtained from local dealers in Aich Prefecture, Japan, in July 2014 and September 2015. Although we examined the wild type of four axolotls (two males and two females), we mainly performed this experiments using albino axolotls. They were kept under laboratory conditions for more than one week before use. Small blocks of chicken liver were fed three times a week, ensuring that no liver remained after feeding. Experiments were performed under the authority of the Animal Care and Use Committee for Fukuoka University of Education, as per the guidelines established by the committee.

2.2. Immunohistochemistry

Eight axolotls (four males and four females) were anesthetized by immersion in a solution of 0.05% ethyl *m*-aminobenzoate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were perfused with 0.65% NaCl, and subsequently with 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB, pH 6.9). The pituitaries were carefully dissected and fixed for one or two days at 4 °C. After washing in PB and immersing in 20% sucrose, the samples were cut into 8-μm-thick sagittal sections with a cryostat (HM525; MICROM, Walldorf, Germany), and thaw-mounted on gelatin-coated glass slides.

Immunohistochemistry was performed as described previously (Suzuki and Yamamoto, 2014a). Briefly, the sections were washed overnight in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS), and incubated with rabbit anti-rat amylin serum (14220-v; Peptide Institute, Osaka, Japan) diluted to 1:1,000 in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT) for 24 h at 4 °C. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratories, Burlingame, Calif., USA) diluted to 1:100 in PBS-BSAT for 1 h at room temperature. The sections were then washed again in PBS and incubated with avidin–biotin–horseradish peroxidase complex (ABC; Vector Laboratories) at a dilution of 1:200 in PBS-BSAT for 30 min at room temperature. After a final wash in PBS, the sections were reacted with 0.02% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris–HCl buffer solution (pH 7.4). Thereafter, the sections were counterstained with thionin, and coverslipped using Malinol (Muto Pure Chemicals, Tokyo, Japan). In some sections, amylin-like immunoreactivity was detected by Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206; Invitrogen Corp., Carlsbad, Calif., USA) and nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Negative controls were prepared by omitting the antiserum in the first incubation or by using antiserum pre-absorbed with synthetic rat amylin (50 μg/ml, 4220-v; Peptide Institute). As another control, the antiserum was also pre-absorbed with synthetic α-melanocyte-stimulating hormone (αMSH) (50 μg/ml, 4057-v; Peptide Institute), because preliminary observations suggested that amylin-immunoreactive cells corresponded to melanotrophs. Double immunofluorescence staining of anti-amylin serum with sheep anti-αMSH antibody (AB5087; Millipore, Temecula, Calif., USA) was performed. Amylin and αMSH (1:10,000) immunoreactivities were visualized using Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206; Invitrogen Corp.) and rhodamine-conjugated donkey anti-sheep IgG (AP184R; Millipore), respectively. To examine the cross-reaction of the anti-αMSH antibody with amylin, some sections were processed using the anti-αMSH antibody pre-absorbed with synthetic rat amylin (50 μg/ml; Peptide Institute) and the immunoreactivity was visualized using DAB.

2.3. Immunoelectron microscopy

The pituitaries dissected from three similarly perfused axolotls (two males and one female) were used for the post-embedding method. Small blocks of the fixed pituitaries were embedded in LR