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Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 261–264, January 1987
Medical Sciences

Evidence for high peptide α-amidating activity in the pancreas from neonatal rats

(thyrotropin-releasing hormone biosynthesis/prohormone processing/Langerhans islets)

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Communicated by Alfred Jost, September 12, 1986 (received for review May 21, 1986)

ABSTRACT **A high peptidylglycine α-amidating monooxygenase (PAMase) activity has been measured in the pancreas of neonatal rats. A significant fraction of this activity is contained in the beta cells of the islets of Langerhans and is colocalized with thyrotropin-releasing hormone (TRH) and its precursor in secretory granules. The ontogenetic variation of PAMase activity in the pancreas parallels that of TRH concentrations, suggesting that this enzymatic activity is directly related to TRH biosynthesis. In addition, PAMase activity is able to generate TRH when incubated with <Glu-His-Pro-Gly, a tetrapeptide present as a repetitive sequence in the TRH precursor. The perinatal evolution of the TRH precursor levels in the pancreas is similar to that of PAMase activity (unpublished results). Thus, the neonatal rat pancreas offers an endocrine model in which the levels of a neuropeptide precursor and an enzyme activity, involved in the posttranslational modification of this precursor, are similarly regulated. Our results suggest also that a fraction of PAMase activity may be produced outside of the beta cells and related to the biosynthesis of COOH-terminally amidated peptide(s) other than TRH. The ontogenetic changes in PAMase activity imply that the synthesis of this peptide(s) is high during the neonatal period, decreasing thereafter.**

Thyrotropin-releasing hormone (TRH, <Glu-His-Pro-NH_2) was initially isolated in the hypothalamus (1, 2); however, its distribution is ubiquitous, including the whole brain and the gastrointestinal tract (3). The ontogenetic pattern of TRH in the pancreas of the rat is peculiar, with the highest levels just after birth, decreasing rapidly toward low levels in the adults (4–6). Despite the early isolation of TRH, its biosynthesis has remained obscure until recently (7). Results from different groups (including ours) support the hypothesis that TRH, like other neuropeptides, arises from the posttranslational processing of a large precursor protein (8, 9). This hypothesis, strongly reinforced by the determination of the cDNA sequence of a portion of the TRH precursor in amphibian skin (10), has been confirmed by the characterization of a cDNA clone coding for the precursor of TRH in the rat hypothalamus (11).

TRH is a COOH-terminally α-amidated tripeptide. Amino acid sequence data for many peptide precursors (including the incomplete sequence of the TRH precursor in amphibian skin) indicate that peptides with COOH-terminal glycine are the intermediate precursors to COOH-terminally α-amidated peptides (12). The mechanisms of the posttranslational modifications of the TRH precursor have not been established. First, they may involve proteolytic cleavages that give rise to the tetrapeptide Gln-His-Pro-Gly. Then, TRH may be generated after the spontaneous cyclization of the NH_2-terminal amino acid and the enzymatic amidation of proline, with the glycine residue acting as the amide donor under the action of a peptidylglycine α-amidating monooxygenase (PAMase). The hypothetical pathway of TRH described above is supported by studies on the maturation of the prooxytocin-neurophysin precursor (13, 14). PAMase activity has been characterized in the intermediate and anterior lobes of the pituitary (15–17), in the mouse pituitary corticotropic tumor cells (At-T20) (18), as well as in the hypothalamus, where it may be involved in the biosynthesis of several bioactive peptides, such as TRH, oxytocin, and vasopressin (19). PAMase activity is barely detectable in the pancreas of the adult rat (20).

In this study, we present evidence that PAMase* activity is high in the rat pancreas during the neonatal period and follows a pattern similar to that of TRH levels in this tissue. Our results suggest also that a significant fraction of pancreatic PAMase activity is involved in the biosynthesis of TRH.

MATERIALS AND METHODS

Animals. Female Long Evans rats were bred in our laboratory and their litters were used in the experiments. The day sperm was identified in the vaginal smear was designated day 0 of pregnancy (gestational length, 22 days) and the day of birth was designated day 0 of life. The animals were housed in individual cages at 23 ± 2°C under controlled light (0700–2000 hr) daily and fed Purina Chow and tap water ad libitum.

Amidation Assay. Amidation activity was measured on crude mitochondrial/secretory granules prepared from freshly dissected pancreata collected from 19-day fetuses as well as from 1- to 90-day-old rats. The pancreata were homogenized at 4°C (in a Teflon/glass homogenizer, 10 strokes) in 10 vol of 0.3 M sucrose/0.02 M Tris·HCl, pH 7.4. The homogenate was centrifuged at 400 × *g* for 10 min at 4°C to sediment nuclei and cell debris. Supernatant was then centrifuged at 12,000 × *g* for 30 min at 4°C. The pellet containing the mitochondrial/secretory granules was resuspended in 0.5 ml of 10 mM phosphate buffer (pH 7.0), submitted to three consecutive freeze-thaw cycles, and stored until assay for amidating activity. The protein concentrations in these preparations were determined according to Bradford (21) using the protein assay kit from Bio-Rad. Since PAMase activity in the pituitary gland is dependent on $CuSO_4$, ascorbate, and molecular oxygen (22), we have tested the influence of all

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Abbreviations: TRH, thyrotropin-releasing hormone; α-MSH, α-melanocyte-stimulating hormone; PAMase, peptidylglycine α-amidating monooxygenase; TRH-OH, <Glu-His-Pro-OH.

*Since peptide α-amidating activity in the pancreas displays the same characteristics as PAMase activity in other tissues, it will be referred to as PAMase in this report.

three agents on PAMase activity from pancreas. The addition of 3 mM ascorbate and 40 μM $CuSO_4$ was optimal for PAMase activity. However, since the optimal amount of $CuSO_4$ to be added in the assay appears to be critical according to the tissue (20), all of the measurements of PAMase activity were performed with various amounts of $CuSO_4$ ranging from 10 to 180 μM. The rate of the α-amidation reaction was also determined in the presence of an air atmosphere (control) or in the presence of a constant stream of helium to replace most of the molecular oxygen.

The PAMase assay was performed at 37°C for 4 hr in a final volume of 0.05 ml using the method of Eipper *et al.* (22). The following reagents were added to each tube: 3 mM ascorbate, 100 μg of catalase per ml; 45 μg of tissue protein; 25 μM D-Tyr-Val-Gly (Novabiochem, Laüfelfingen, Switzerland), ^{125}I-labeled D-Tyr-Val-Gly (18,000–20,000 cpm), and increasing amounts of $CuSO_4$ (10–180 μM). All of the reagents were prepared in 0.12 M sodium *N*-[tris(hydroxymethyl)methyl]-aminoethanesulfonic acid (pH 7.4) (Merck, Darmstadt, F.R.G.). The labeled substrate and product were separated on a 1-ml column of sulfopropyl Sephadex C25-120 (Pharmacia, Uppsala) equilibrated in 10 mM phosphate buffer (pH 5.0). After a 12-ml wash in the same buffer, elution was performed with 0.5 M NaCl in 0.05 M phosphate buffer (pH 5.0). The α-amidation activity was directly related to the rate of conversion of ^{125}I-labeled D-Tyr-Val-Gly into ^{125}I-labeled D-Tyr-Val-NH_2 and was expressed as pmol/hr per μg of protein of D-Tyr-Val-NH_2 generated from D-Tyr-Val-Gly. All samples were assayed in duplicate and generally deviated <5% from the mean.

Determination of the Conversion Rate of <Glu-His-Pro-Gly into <Glu-His-Pro-NH_2 (TRH) by Neonate Rat Pancreatic Preparation. As suggested in the Introduction, the tetrapeptide <Glu-His-Pro-Gly is a putative natural substrate for PAMase activity in pancreatic secretory granules. Thus, it was of interest to test whether pancreatic PAMase was able to convert <Glu-His-Pro-Gly into TRH. The tetrapeptide <Glu-His-Pro-Gly (synthesized for us by J. Van Rietschoten and C. Granier, Institut National de la Sánte et de la Recherche Médicale, U 172, Marseille, France) (25 μM) was incubated with a pancreatic mitochondrial/secretory granule preparation from 3-day-old rats under optimal assay conditions at 37°C for 0–4 hr. The reaction was stopped by the addition of 0.5 ml 5 M acetic acid, followed by boiling for 10 min. The samples then were centrifuged at +4°C (2000 × *g*, 10 min) and the supernatant was evaporated to dryness. TRH levels in the tubes were determined by radioimmunoassay (23).

Cellular Localization of PAMase Activity in the Rat Pancreas. To study the cellular localization of PAMase activity in the neonate rat pancreas, 1-day-old rats were injected with streptozotocin, a drug displaying specific cytotoxicity toward the beta cells of the endocrine pancreas (24). Streptozotocin (Sigma) was dissolved extemporaneously in 0.1 M sodium citrate (pH 4.5) and injected i.p. into 1-day-old rats (150 mg/kg). Control rats received the same volume (0.1 ml) of the vehicle. At the age of 4 and 6 days, the rats were sacrificed, and their pancreata were collected and prepared for measurement of PAMase activity. In the group of streptozotocin-treated rats, only the pancreata from rats with glycemia >3.50 g/liter were used for the experiment.

Subcellular Localization of PAMase Activity in the Neonate Rat Pancreas. A pancreatic mitochondrial/secretory granule preparation was obtained as described, except that the initial homogenization was performed after exposure to DNase I (26 μg/ml at room temperature for 5 min). This preparation was layered on the top of 11 ml of 33.3% isoosmotic Percoll (Pharmacia, Upsala) in 0.3 M sucrose/0.02 M Tris·HCl, pH 7.4. After centrifugation at +4°C (65,000 × *g*, 60 min), 0.4-ml fractions were collected by puncturing the tubes and were assayed for PAMase activity (22), TRH (23), and TRH precursor (9).

Measurement of TRH and Its Precursor. TRH levels were determined by a radioimmunoassay (23) in subcellular fractions from 3-day-old rat pancreas and in extracts from rat pancreata obtained at different developmental stages. Pancreatic extracts were prepared as follows. Freshly dissected rat pancreata were boiled for 15 min in 10 vol of 5 M acetic acid, cooled on ice, homogenized in a Teflon/glass homogenizer (10 strokes), and centrifuged at 2500 × *g* (4°C, 15 min). The acidic and clear supernatant was evaporated to dryness in a Speed Vac apparatus (Savant, Hicksville, NY). The TRH antiserum shows 1% crossreaction with <Glu-His-Pro-Gly and no crossreaction (<0.01%) with the putative metabolites of TRH, <Glu-His-Pro-OH (TRH-OH), and histidylproline-diketopiperazine.

The levels of TRH precursor were determined in the subcellular fractions from 3-day-old rat pancreas as described by Ouafik *et al.* (9). Briefly, the samples were appropriately diluted and subjected to a sequential enzymatic treatment by L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (50 μg/ml) for 2 hr at 37°C (Worthington) and carboxypeptidase A (Sigma) for 2 hr at 37°C. The enzymes were successively heat-inactivated (95°C, 20 min). The sequence TRH-OH generated by the enzymatic treatment of the precursor for TRH was measured by a specific radioimmunoassay (6). TRH precursor levels were expressed as the difference between the values of TRH-OH-immunoreactive material measured in the different fractions after and prior to sequential enzymatic treatment. The antiserum anti-TRH-OH shows no significant crossreaction with TRH and <Glu-His-Pro-Gly (<0.0001).

TRH and TRH-OH used as standards in the radioimmunoassays have been purchased from Sigma.

RESULTS

Preliminary Characterization of PAMase Activity in the Neonate Rat Pancreas. The crude mitochondrial/secretory granule preparations from neonatal rats displayed a high PAMase activity. This activity was linear in time for up to 4 hr and linear in content of protein for up to 170 μg (results not shown).

This activity, like enzymatic PAMase activity described in other tissues (22), required the presence of $CuSO_4$, ascorbate, and molecular oxygen and is therefore referred to as pancreatic PAMase in the rest of the text.

Ascorbate requirement was the same as in other tissues, but the optimal concentration of $CuSO_4$ in pancreatic preparations (close to 40 μM) was four and eight times higher than in the anterior (10 μM) and neurointermediate (5 μM) lobe of the pituitary gland, respectively. $CuSO_4$ concentrations >50 μM resulted in an inhibition of this activity. In the absence of molecular oxygen in the reaction mixture, there was a 60% decrease of the pancreatic PAMase activity. The velocity of the enzymatic reaction appeared to be of the same order of magnitude in the 3-day-old rat pancreas (4 pmol/hr per μg of protein) and in the adult rat pituitary gland (10 and 2 pmol/hr per μg of protein in the anterior and neurointermediate lobe, respectively).

Ontogenetic Evolution of Pancreatic PAMase Activity. As shown in Fig. 1 [and as published by different groups (4–6)], immunoreactive TRH concentrations in the rat pancreas reached a peak 4 days after birth and then decreased rapidly toward the minute levels found in adults. PAMase activity was already detectable in the pancreata from 19-day fetuses. The highest PAMase activity was observed in 3-day-old rats. Thereafter, PAMase activity decreased rapidly with a 50% reduction between the third and fourth day of life. Then a plateau was reached between the fifth and eighth day;

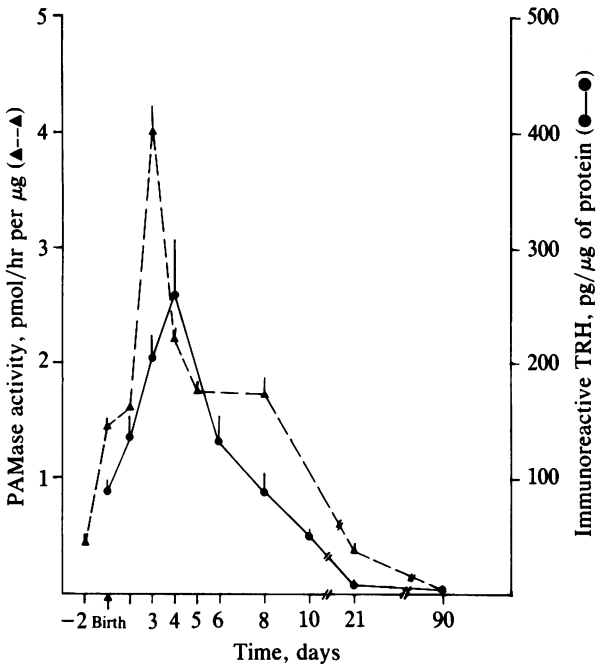

FIG. 1. Ontogenesis of PAMase (▲- -▲) activity and TRH (●—●) levels in rat pancreas. The PAMase activity was measured under the conditions described in the text, using a constant amount of pancreatic mitochondrial/secretory granule preparation (45 μg of protein) and $CuSO_4$ (40 μM) at all stages investigated. TRH was measured by radioimmunoassay in pancreatic extracts as described by Dutour *et al.* (6). The results are expressed as mean ± SEM (six to eight determinations at each developmental stage).

thereafter, a further decrease was observed. When the rats were 21 days old, the PAMase activity in the pancreas represented 9% of the activity observed in 3-day-old rats. In adult rats, this activity was very low, barely detectable (<0.05 pmol/hr per μg of protein). The peak of PAMase activity preceded that of TRH by 1 day. Then TRH levels decreased more rapidly than PAMase activity.

Ability of Pancreatic PAMase Activity to Convert the Tetrapeptide <Glu-His-Pro-Gly into TRH. As shown in Table 1, high levels of immunoreactive TRH were generated after incubation of <Glu-His-Pro-Gly with the mitochondrial/secretory granule preparation, indicating that the high PAMase activity in the pancreas of 3-day-old rats may be responsible for the high rate of synthesis of TRH at this stage. However, the levels of TRH generated under these conditions were lower than that expected on the basis of the conversion rate of ^{125}I-labeled D-Tyr-Val-Gly into ^{125}I-labeled D-Tyr-Val-NH_2. Indeed, in 3-day-old rats, the amidation activity was lower by a factor of 7.8 when measured with <Glu-His-Pro-Gly rather than D-Tyr-Val-Gly as a substrate.

Table 1. Generation of TRH after incubation of crude mitochondrial/secretory granule preparation from 3-day-old rat pancreas with the tetrapeptide <Glu-His-Pro-Gly

Incubation conditions	TRH content, fmol of TRH per tube
Without tetrapeptide, 0 min	8 ± 2.17
With tetrapeptide	
0 min	76 ± 21.6
4 hr	2240 ± 134

Incubation times are indicated. Results are expressed as mean ± SEM of four determinations. The increased TRH concentrations after addition of <Glu-His-Pro-Gly and before incubation are due to the crossreactivity of the TRH antiserum with the tetrapeptide.

Table 2. Effect of streptozotocin treatment on the pancreatic PAMase activity of 4- and 6-day-old rats

Age, days	PAMase activity, pmol/hr per μg of protein: Control	Streptozotocin-treated
4	2.5 (6)	1.27 (6)
6	1.17 (5)	0.52 (4)

D-Tyr-Val-NH_2 was generated from D-Tyr-Val-Gly. Results are expressed as the mean of two determinations. The difference between extreme values and the mean was <5%. The number of animals per determination is given in parentheses.

Cellular Localization of Pancreatic PAMase Activity. As shown in Table 2, the treatment with streptozotocin induced a 50% and 55% decrease of PAMase activity in 4- and 6-day-old rats, respectively. Since streptozotocin toxicity is specific for beta cells, these data suggest that about one-half of the PAMase activity is present in these cells.

Subcellular Localization of Pancreatic PAMase Activity. After subcellular fractionation of 3-day-old rat pancreas homogenates over an isoosmotic Percoll density gradient, the distributions of PAMase activity and of TRH and TRH precursor (the putative product and substrate of pancreatic PAMase, respectively) were shown to display similar profiles, characterized by a single peak containing >80% of these activities (Fig. 2). This peak corresponded to organelles of density between 1.06 and 1.08 and also contained most of insulin immunoreactivity (results not shown).

DISCUSSION

TRH concentrations in the pancreata from neonatal rats are very high during a short, transient period (4–6). The physiological role of TRH in the pancreas of neonates is not understood; however, the rapid changes in TRH levels in the rat pancreas during the neonatal period make this tissue an interesting model for studying the mechanisms and regulation of TRH biosynthesis. Pancreata from adult rats that display low levels of TRH contain very low, barely detectable

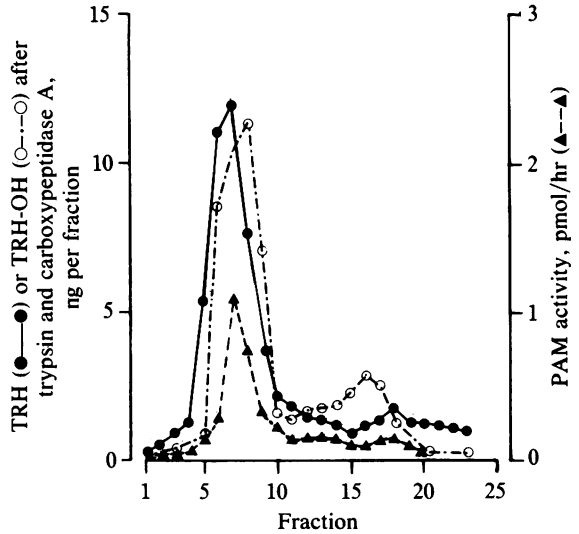

FIG. 2. PAMase (▲- -▲) activity and TRH (●—●) and TRH precursor (○-·-○) levels in 4-day-old rat pancreatic mitochondrial/secretory granule preparation fractionated on Percoll gradients. The 0.4-ml fractions were assayed for PAMase activity according to Eipper *et al.* (22) and for TRH (23) and TRH precursor (9). TRH precursor levels are expressed as ng of TRH-OH generated after sequential enzymatic treatment with trypsin and carboxypeptidase A.

amounts of PAMase activity (20). Pancreata from neonatal rats contain very high PAMase activity, which peaks on the third postnatal day. The ontogenetic evolution of PAMase activity in the pancreas is parallel to that of TRH in this tissue, suggesting a direct relationship between both substances. The colocalization of TRH, its precursor, and PAMase activity in the subcellular fractions of pancreatic tissue adds further evidence for a role of PAMase activity in the biosynthesis of TRH. In addition, pancreatic PAMase activity is able to generate TRH, when incubated with <Glu-His-Pro-Gly, a tetrapeptide that has been identified as a repetitive sequence present in the TRH precursor (11). This tetrapeptide has been utilized as a substrate to demonstrate the capacity of hypothalamic and hypophysial PAMase to generate TRH (19, 25, 26).

The reduction of PAMase activity in the pancreata of streptozotocin-injected rats has two implications. First, it suggests that, like insulin and TRH (27, 28), part of this enzymatic activity is contained in the beta cells of the islets of Langerhans. Second, it indicates also that a significant fraction of PAMase activity is present in pancreatic tissue outside of the beta cells, in other cells of the islets of Langerhans and/or in exocrine pancreas. Indeed, peptide amidation activity has been described in exocrine tissues, including the rat exocrine pancreas (29). PAMase activity may be directed toward proteins other than TRH precursor in the pancreas. This activity has been assumed to be involved in the amidation process of various pituitary peptides, such as α-melanocyte-stimulating hormone (α-MSH), oxytocin, and vasopressin. PAMase activity in the pancreas may have also a broad specificity and be involved in the synthesis of other peptides. The list of peptides ending with amidated residues and present in the pancreas includes vasoactive intestinal polypeptide, secretin, gastrin-releasing peptide, neuropeptide Y, pancreatic polypeptide (PP), and gastrin. Detailed information on the postnatal evolution of all of these peptides is not yet available. However, the evolution of gastrin levels in the neonate rat pancreas strikingly follows a pattern similar to that of TRH (30). The number of PP-storing cells increases abruptly in 5- to 7-day-old rats (31). Interestingly, the plateau of PAMase activity between the fifth and seventh day occurs during the period of maximum increase of PP cell number.

Other data from our group indicate that the ontogenetic evolution of TRH precursor in the pancreas parallels that of PAMase activity and TRH (unpublished results). Thus, it appears that the levels of a neuropeptide precursor (TRH precursor) and of an enzymatic activity (PAMase activity) involved in the posttranslational modifications of this precursor are similarly regulated in the neonate rat pancreas. The COOH-terminally amidated peptide, α-MSH is normally produced by the neurointermediate lobe of the pituitary gland through the processing of proopiomelanocortin by several proteolytic enzymes, acetylating enzyme, and PAM activity. It has been reported that glucocorticoids decrease proopiomelanocortin levels and PAMase activity in At-T20 cells (32). No information is available on the effects of glucocorticoids on the activity of other enzymes involved in the processing of proopiomelanocortin. It must be noted that At-T20 cells do not synthesize α-MSH, probably because proteolytic cleavages of proopiomelanocortin do not give rise to a suitable substrate for PAMase activity in these cells. Hence, the function of PAMase activity in At-T20 cells is not yet determined. Nevertheless, it is interesting to note that the syntheses of peptide precursors and PAMase activity are coregulated in two different tissues.

It has been shown that bovine neurointermediate pituitary contains two forms of PAMase activity that differ in apparent molecular weight (33). Further studies are needed to characterize and purify PAMase in the pancreas. These investigations would allow the determination of the reaction mechanism of this enzyme and the development of antibodies and cDNA probes, and hence the ability to study the localization and regulation of this (these) enzyme(s).

We are greatly indebted to Dr. J. Van Rietshoten and Dr. C. Granier (Institut National de la Santé et de la Recherche Médicale, U 172, Marseille) for generously providing synthetic <Glu-His-Pro-Gly. We also thank Ms. R. Querat for her expert secretarial assistance and Ms. R. M. Saura for her technical help.

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