Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 179-184

EVIDENCE FOR HIGH PEPTIDE α-AMIDATION ACTIVITY IN THE NEONATAL RAT PANCREAS

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Received May 6 , 1986

SUMMARY : A high peptide α-amidating activity is present in a mitochondrial/secretory granules preparation from 3-day old rat pancreas. It is dependent on copper, ascorbate and molecular oxygen. This preparation is able to generate TRH when incubated with Pyroglu-His-Pro-Gly, a sequence present in the TRH precursor molecule. The peptide α-amidating activity may be involved in the high rate of TRH biosynthesis in the pancreas during the neonatal period. In the pancreas of adult rats which contain low levels of TRH, the peptide α-amidating activity is barely detectable.

The tripeptide Pyroglutamyl-Histidine-Prolineamide (Thyrotropin-Releasing Hormone, TRH) initially identified in mammalian hypothalamus, is also present in other parts of the body including the pancreas (1). Like many other bioactive peptides, TRH terminate with a carboxyl-terminal α-amide moiety. The enzymatic activity involved in the synthesis of amidated product peptides (-X-NH2) from appropriate precursor peptides (-X-Gly) has been initially identified in pig pituitary secretory granules using the synthetic substrate D-Tyr-Val-Gly (2). Such an enzymatic activity has been detected in several tissues and body fluids from the rat including pituitary, hypothalamus, submandibular gland, thyroid, antrum, serum and cerebrospinal fluid. However, this activity is very low, barely detectable in the adult rat pancreas (3). TRH levels change rapidly in the rat pancreas

0006-291X/86 $1.50

during the neonatal period reaching a peak in 4-day old rats, then returning to the low, adult levels after ten days (4,5). The synthesis of TRH involves the processing of a high molecular weight peptide precursor (6) under the action of several enzymes including a peptide α-amidating enzyme(s). Thus, it was of interest to test whether this enzymatic activity was present in the neonate rat pancreas and related to TRH biosynthesis.

METHODS AND MATERIALS

- Tissue extraction :
Female rats of the Long-Evans strain were bred in our laboratory. Pancreas from 3-day old and adult rats (b.w. 275g) were rapidly removed and immersed in 0.3 M Sucrose, 0.02 M Tris-Hcl, pH 7.4 at 4°C (1:10, w/v). The mitochondrial/secretory granules preparation was obtained as follows. After homogenization with a Teflon-Glass homogenizer (10 strokes), the tissue suspension was centrifuged at 400xg for 10 min at 4°C in order to sediment nuclei and cell debris. The supernatant was then centrifuged at 10,000xg for 30 min at 4°C. The pellet was resuspended in 0.5 ml 0.01 M Phosphate Buffer pH 7.0., submitted to three consecutive freeze-thaw cycles and stored until assayed for peptide α-amidating activity. The protein concentration in the preparations was determined according to Bradford using the Protein Assay kit from Biorad (7).
- Measurement of peptide α-amidating activity :
The determination of peptide α-amidating activity was performed at 37°C for 4 hours according to Eipper et al. (8). The reaction mixture (0.05 ml) contained : - crude pancreatic mitochondrial/secretory granules (equivalent to 45 ug protein) - 25 uM D-Tyr-Val-Gly (Novabiochem, Switzerland) - [125 I] D-Tyr-Val-Gly (prepared in our laboratory using the chloramin T method (9)), 18 to 20,000 cpm - 100 ug/ml catalase - increasing amounts of CuSO4 (10 to 180 uM) - increasing amounts of ascorbate (0 to 10 mM). All the reagents were prepared in 0.12 M Na N-Tris-[hydroxymethyl] methyl-2-aminoethane sulfonic acid (TES) pH 7.4 (Merck, Darmstadt, FRG). The peptide α-amidating activity was directly related to the rate of conversion of [125 I] D-Tyr-Val-Gly into [125 I] D-Tyr-Val-NH2. The labeled substrate and product were separated on a 1 ml column of Sulphopropyl Sephadex C25-120 (Pharmacia, Upsala, Sweden) equilibrated with 10 mM Phosphate Buffer, pH 5.0. After a 12 ml wash with the same buffer, the elution was performed with 0.5 M NaCl, 0.05 M Phosphate Buffer, pH 5.0. All samples were assayed in duplicate and generally deviated less than 5% from the mean. The peptide α-amidating activity was expressed as pmol/h/ug prot. of D-Tyr-Val-NH2 generated from D-Tyr-Val-Gly
- Conversion of [125 I]-Pyroglu-His-Pro-Gly into [125 I]-TRH:
[125 I]Pyroglu-His-Pro-Gly (labeled in our laboratory using the chloramin T method) was incubated at 37°C for different

periods of time (0 to 4 hours) with a crude mitochondrial/secretory granules preparation from 3-day old rat pancreas. The incubation mixture contained : - the pancreatic preparation (equivalent to 60 ug protein) - [125I] Pyroglu-His-Pro-Gly 150,000 cpm - 25 uM Pyroglu-His-Pro-Gly - 100 ug/ml catalase - 40 uM CuSO4 - 3 mM ascorbate.
The reaction was stopped by the addition of 0.5 ml 0.2 M Phosphate Buffer pH 7.4. Then, the reaction mixture was run over an anti-TRH immunoaffinity column. This column was prepared by conjugating the purified immunoglobulin fraction from an anti-TRH antiserum to the reactive groups of an ultraaffinity - EP column (50 x 34.6 mm) according to the instructions provided by the manufacturer (Beckman). After washing with 10 ml 0.2 M Phosphate Buffer pH 7.4, the [125 I] TRH was eluted from the column with 2 N acetic acid. The total radioactivity in the fractions containing acetic acid eluates was determined in a gamma counter and served as an estimation of [125 I] TRH. The recovery of [125 I] TRH from the column was 65%. No degradation of [125 I] TRH was observed after a 4 hours incubation with the pancreatic preparation.

RESULTS

A high peptide α-amidating activity was demonstrated in a crude mitochondrial secretory granules preparation from 3-day old rat pancreas. This activity was dependent on molecular oxygen (results not shown) and stimulated by the addition of 3 mM ascorbate. Its dependence on copper is shown in Figure 1. Under optimal concentration of CuSO4 (40 uM) and Ascorbate

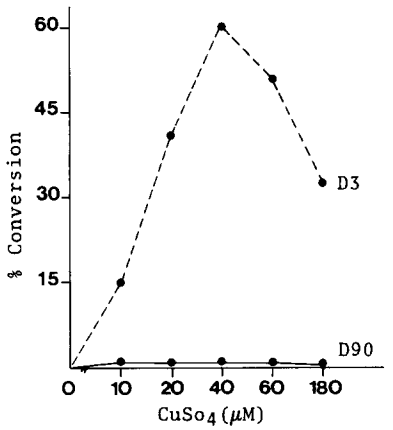

Fig. 1 : Copper-dependence of peptide α-amidating activity in the pancreas of 3-day old (•----•) and adult rats(•——•). The incubation was carried out at 37°C for 4 hours. The tubes contained : increasing concentrations of CuSO4, [125 I]-D-Tyr-Val-Gly pancreatic crude mitochondrial/secretory granules (equivalent to 45 ug protein), and 120 mM sodium TES, pH 7.4.

Table 1 : Conversion of [125 I]-Pyroglu-His-Pro-Gly into [125 I]-TRH after incubation with a mitochondrial/secretory granules preparation from 3-day old rat pancreas

Incubation time (hour)	TRH generated (pmol/h/ug prot)
1	0.379
2	0.742
4	1.08

(3 mM), the velocity of the enzymatic reaction was 4 pmol/h/ug protein. The peptideα-amidating activity was very low, barely detectable (<0.05 pmol/h/ug protein) in the same preparation from adult rats. The incubation of [125 I] Pyroglu-His-Pro-Gly with the same preparation from 3-day old rats resulted in the formation of [125 I] TRH. The conversion of [125 I] Pyroglu-His-Pro-Gly into [125 I]-TRH was linear in time at least for up to 2 h and its velocity was closed to 0.370 pmol/h/ug protein (Table 1).

DISCUSSION

The present study is the first evidence for the presence of a peptide α-amidating activity in the rat pancreas. Indeed, this activity is very high in the pancreas from 3-day old rats. It has similar properties to the peptidyl-glycine characterized in the rat hypothalamus (10) and neurointermediate pituitary secretory granules (11). Indeed, this enzymatic activity is dependant on copper, ascorbate and molecular oxygen. The optimal amount of CuSO4 to add to pancreatic extracts is higher (40 uM) than in the adult rat anterior lobe (10 uM) and neurointermediate lobe (5 uM). The addition of an optimal level of copper sulfate may reveal or increase PAM activity by eliminating the effect of endogenous inhibitors present in tissue extracts. Then, the levels of

these endogenous inhibitors may be higher in the pancreas than in other tissues. The peptide α-amidating activity in the pancreas may be referred as PAM and belongs to a group of enzymes depending on copper, ascorbate and molecular oxygen, such as dopamine ß-hydroxylase (12).

The high PAM activity in the neonate rat pancreas may be responsible for the accelerated TRH biosynthesis observed during the neonatal period. Indeed, the mitochondrial/ secretory granules preparation from 3-day old rats is able to convert the tetrapeptide Pyroglu-His-Pro-Gly into TRH. This tetrapeptide is contained into the sequence of the molecule of TRH precursor recently identified in the rat hypothalamus (6). It is likely generated during the processing of the TRH precursor and may be the direct substrate for PAM activity. Furthermore, it has previously been shown that PAM activity from rat brain (13) and beef posterior pituitary (14) is capable of converting Pyroglu-His-Pro-Gly into authentic TRH. However,PAM activity in the neonate rat pancreas may also be involved in the biosynthesis of carboxy-terminus α-amidated peptides other than TRH, such as Gastrin, Vasointestinal Active Peptide, Corticotropin-Releasing Factor and Pancreatic Polypeptide (PP). Full informations on the postnatal evolution of all these peptides are not available yet. Interestingly, the evolution of Gastrin levels in the neonate rat pancreas follows a similar pattern to that of TRH (15) and the number of PP storing cells increases abruptly in the pancreas of 5 to 7-day old rats (16).

ACKNOWLEDGMENTS

Supported by grants from Nestlé-France, C.N.R.S. (UA 560) and Fondation pour la Recherche Médicale Française. The authors are indebted to Dr A. Cupo (INSERM-CNRS Centre d'Immunologie de Marseille) for having purified the immunoglobulin fraction from anti-TRH antiserum and to Drs J.

Van Rietschoten and C. Granier (INSERM U172) for the synthesis of Pyroglu-His-Pro-Gly. The authors thank Ms R. Querat for her excellent secretarial assistance.

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